

M.H



00/786972  
PCT/AU99/00762

AU 99/762

REC'D 19 OCT 1999	
WIPO	PCT

EJHV

Patent Office  
Canberra

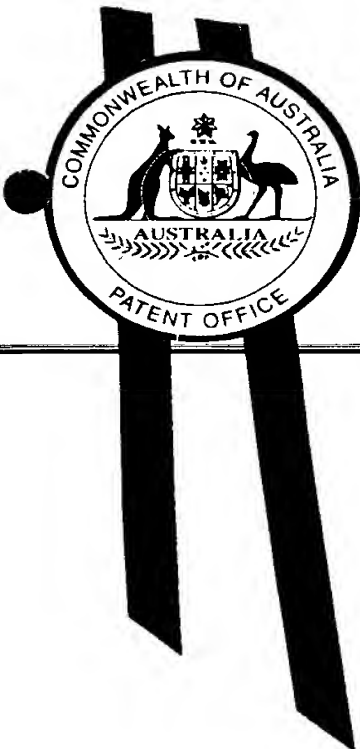
I, KIM MARSHALL, MANAGER PATENT OPERATIONS hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PP 5843 for a patent by STARPHARMA PTY LTD filed on 14 September 1998.

I further certify that the name of the applicant has been amended to STARPHARMA LIMITED pursuant to the provisions of Section 104 of the Patents Act 1990.

WITNESS my hand this  
Thirteenth day of October 1999

A handwritten signature in dark ink, appearing to be 'Kim Marshall'.

KIM MARSHALL  
MANAGER PATENT OPERATIONS



**PRIORITY  
DOCUMENT**  
SUBMITTED OR TRANSMITTED IN  
COMPLIANCE WITH RULE 17.1(a) OR (b)

THIS PAGE BLANK (USPTO)

Starpharma <sup>Limited</sup>  
~~Pty. Ltd.~~

A U S T R A L I A  
Patents Act 1990



PROVISIONAL SPECIFICATION  
for the invention entitled:

"Inhibition of toxic materials or substances".

---

The invention is described in the following statement:

- 2 -

## SUMMARY OF THE INVENTION

According to the present invention, there is provided a method of prophylactic or therapeutic inhibition of a toxic material or substance in a human or non-human animal patient, which comprises administration to the patient of an effective amount of a  
5 dendrimer having a plurality of terminal groups wherein at least one of said terminal groups has an anionic- or cationic-containing moiety bonded or linked thereto.

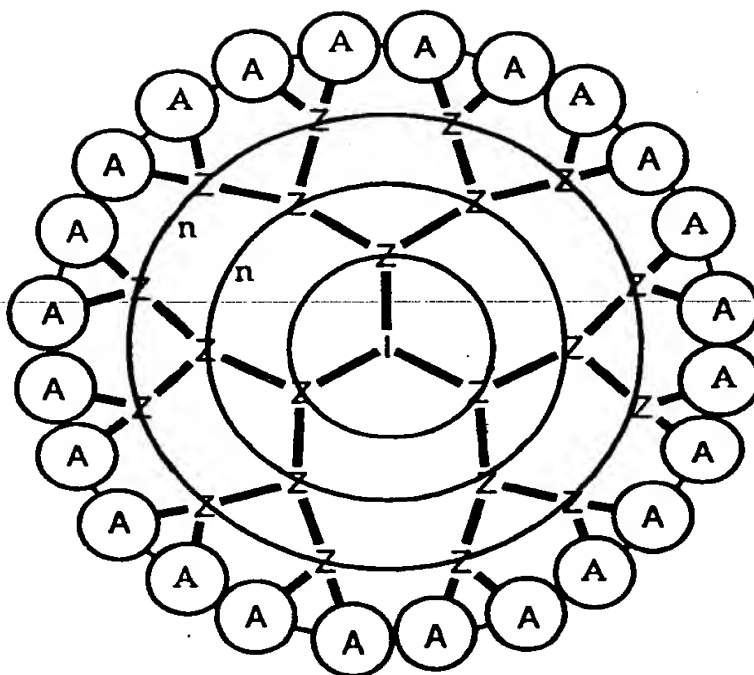
Particularly preferred compounds for use in the method of the present invention  
10 are dendrimers having sulfonic acid-containing moieties, carboxylic acid-containing moieties, phosphoric or phosphonic acid-containing moieties, boronic acid-containing moieties, neuraminic or sialic acid-containing moieties or moieties containing modified neuraminic or sialic acid; primary, secondary, tertiary or quaternary amino-containing moieties, pyridinium-containing moieties; guanidinium-containing moieties;  
15 amidinium-containing moieties; phenol-containing moieties; heterocycles possessing acidic or basic hydrogens; zwitterionic-containing moieties; or mixtures of the above moieties, linked to terminal groups thereof.

The compounds used in the method of this invention are referred to herein as  
20 polyionic dendrimers, and this term is used throughout this specification and the claims which follow to include not only the dendrimers *per se*, but also their pharmaceutically or veterinarily acceptable salts, for example the alkaline metal or alkaline earth metal salts such as the sodium, potassium or calcium salts, as well as pharmaceutically acceptable  
anions such as fluoride, chloride, bromide, iodide, citrate, acetate, p-toluene sulfonate,  
25 and the like.

## DETAILED DESCRIPTION OF THE INVENTION

Preferred compounds used in accordance with the present invention include  
30 polyionic dendrimers of the general formula I:

- 3 -



wherein:

I is an initiator core;

Z is an interior branching unit;

n is an integer which represents the number of generations of the dendrimer; and

A is an anionic- or cationic-containing moiety which may be linked to interior branching unit Z through an optional linking group X.

Dendrimers are macromolecular highly branched compounds formed by reiterative reaction sequences starting from an initial, core molecule with successive layers or stages

being added in successive "generations" to build up a three-dimensional, highly ordered polymeric compound. Dendrimers are characterised by the following features: I an initiator core(I) which may have one or more reactive sites and be point-like or of significant size so as to effect the final topology of the dendrimer; ii layers of branched repeating units (Z) attached to the initiator core; iii functional terminal groups (such as moieties A) attached to the surface of the dendrimer, optionally through linking groups (such as linking groups X).

The present invention uses dendritic structures as frameworks for the attachment of ionic moieties; the invention is not limited to the spherical dendrimers described in detail herein

- 4 -

but can be based on any dendritic structure. The variety of dendrimers in both shape and constitution are well known to persons skilled in the art.

The preparation of dendrimers is well known, and is described by way of example in  
5 U.S. Patents Nos. 4,289,872 and 4,410,688 (describing dendrimers based on layers of lysine units), as well as U.S. Patents Nos. 4,507,466, 4,558,120, 4,568,737 and 4,587,329 (describing dendrimers based on other units including polyamidoamine or PAMAM dendrimers). The dendrimers disclosed in these US patents are described as being suitable for uses such as surface modifying agents, as metal chelating agents, as demulsifiers or  
10 oil/water emulsions, wet strength agents in the manufacture of paper, and as agents for modifying viscosity in aqueous formulations such as paints. It is also suggested in U.S. Patents Nos. 4,289,872 and 4,410,688 that the dendrimers based on lysine units can be used as substrates for the preparation of pharmaceutical dosages.

15 International Patent Publications Nos. WO 88/01178, WO 88/01179 and WO 88/01180 disclose conjugates in which a dendrimer is conjugated or associated with another material such as a carried pharmaceutical or agricultural material. In addition, International Patent Publication No. WO 95/24221 discloses dendritic polymer conjugates composed of at least one dendrimer in association with a carrier material which can be a biological  
20 response modifier, and optionally a target director. These patent publications together with the U.S. patents mentioned above contain a broad disclosure of various dendrimers and processes for the preparation thereof, and the disclosure of each of these publications is incorporated herein by reference.

25 The term "dendrimer" as used herein is to be understood in its broadest sense, and to include within its scope all forms and compositions of these dendrimers as disclosed in Patent Publications Nos. WO 88/01178, WO 88/01179 and WO 88/01180. The term also includes linked or bridged dendrimers as disclosed in these patent publications.

30 The preferred dendrimers of the present invention comprise a polyvalent core

- 5 -

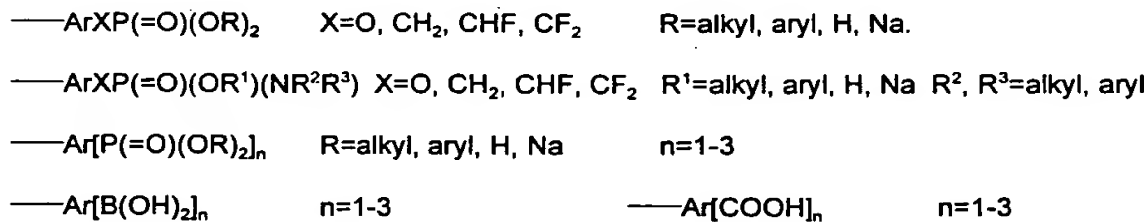
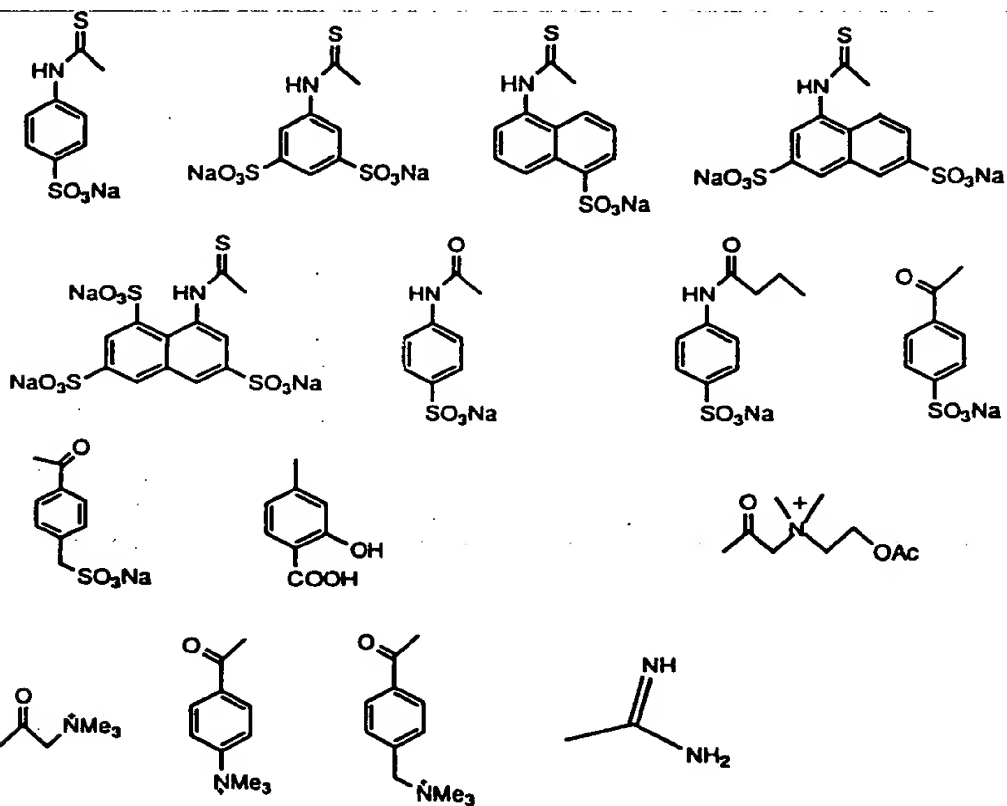
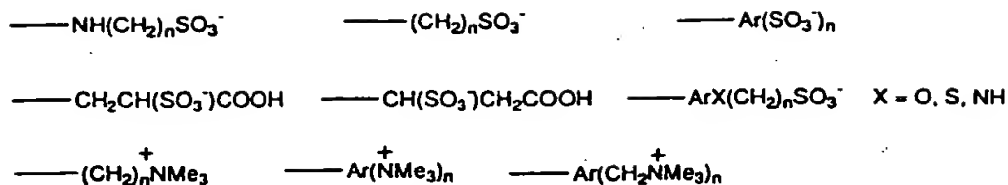
covalently bonded to at least two dendritic branches, and preferably extend through at least two generations. Particularly preferred dendrimers are polyamidoamine (PAMAM) dendrimers, PAMAM (EDA) dendrimers and polylysine dendrimers.

5 In accordance with the present invention, at least one, and preferably a substantial number, of the terminal groups on the surface of the dendrimer has an anionic- or cationic- containing moiety covalently bonded thereto. The branches of the dendrimer may terminate in amino groups or other functional reactive groups such as OH, SH, or the like, which subsequently can be reacted with the anionic or cationic moieties. Where the terminal  
10 groups of the dendrimer are amine groups, the anionic- or cationic-containing moiety may be linked to the dendrimer by a variety of functional groups including amide and thiourea linkages. Preferred anionic- or cationic-containing moieties which may be bonded to the terminal groups of the dendrimer include sulfonic acid-containing moieties, carboxylic acid-containing moieties (including neuraminic and sialic acid-containing moieties and modified  
15 neuraminic and sialic acid-containing moieties), boronic acid-containing moieties, phosphoric and phosphonic acid-containing moieties (including esterified phosphoric and phosphonic acid-containing moieties) and primary, secondary, tertiary or quaternary amino-containing moieties, pyridinium-containing moieties; guanidinium-containing moieties; amidinium-containing moieties; phenol-containing moieties; heterocycles  
20 possessing acidic or basic hydrogens; zwitterionic-containing moieties; or mixtures of the above moieties.

---

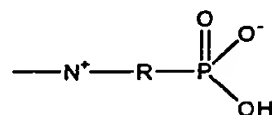
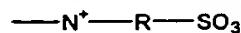
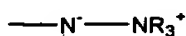
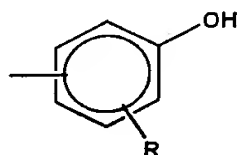
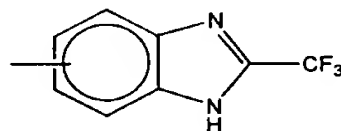
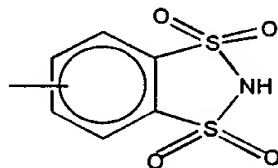
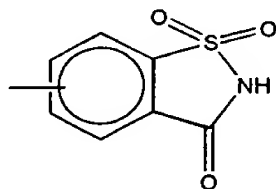
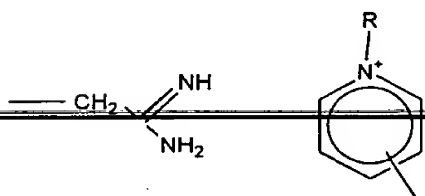
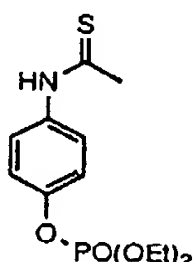
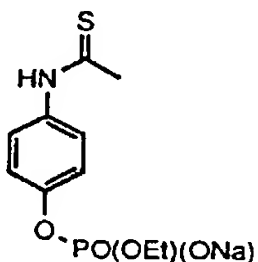
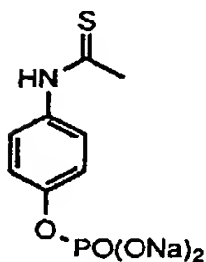
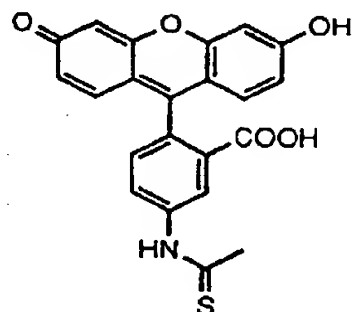
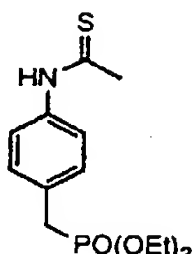
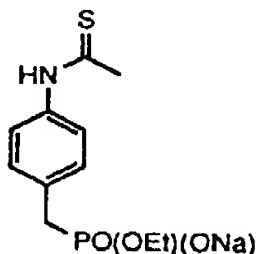
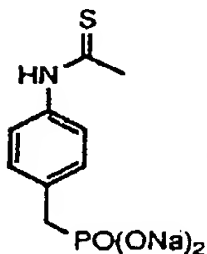
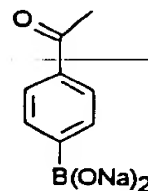
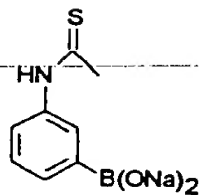
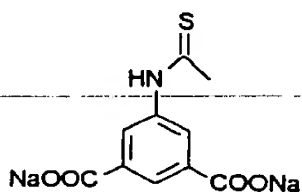
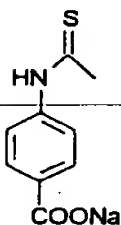
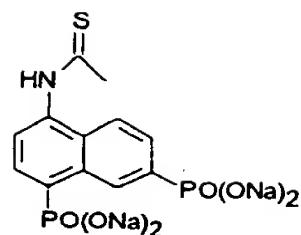
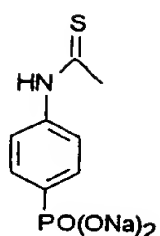
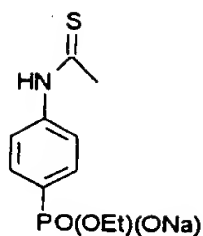
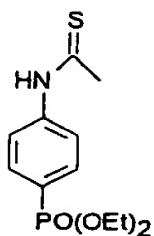
Suitable anionic- and cationic-containing moieties which may be bonded or linked to the amino or other terminal groups include, by way of example, the following groups (in  
25 which n is zero or a positive integer, more particularly n is zero or an integer of from 1 to 20):

- 6 -





- 7 -



- 8 -

In addition to the above, various neuraminic or sialic acid-containing moieties or modified neuraminic or sialic acid-containing moieties may be bonded or linked to the dendrimers in accordance with this invention. These moieties include the various N- and O-substituted derivatives of neuraminic acid, particularly N- and O-acyl derivatives such as N-acetyl, O-acetyl and N-glycolyl derivatives, as well as moieties in which the neuraminic acid group is modified.

Preferably, in the polyionic dendrimers of the general formula I, n is an integer of from 1 to 20 or more, more preferably from 1 to 10. Preferably also, the dendrimers include at least three or more terminal groups.

The optional linking groups which may be present to act as a spacer between the dendrimer and the moiety A, may consist of an alkyl chain (optionally substituted or branched), an alkoxy, polyalkoxy, alkylthio or polyalkylthio chain (optionally substituted), or an alkenyl, multiple alkenyl, alkynyl or multiple alkynyl chain (optionally substituted). Suitable spacer chains include groups of the formula  $-(CH_2)_m-Z-(CH_2)_m-$ , wherein Z is  $-CH_2-$ ,  $-CH=CH-$ ,  $-C\equiv C-$ ,  $-O-$  or  $-S-$ , and m is an integer of from 1 to 15.

The anionic or cationic dendrimers of this invention may be prepared by standard chemical methods which are well known to persons skilled in this art. Suitable methods are described by way of the example in Examples below.

---

~~As previously described, the anionic or cationic dendrimers of the present invention~~ have been found to inhibit toxic materials or substances. The term "toxic materials or substances" as used herein is intended to refer in particular to toxins of biological (animal, plant, microbial or viral) origin, including but not limited to animal toxins or toxic peptides such as snake, scorpion, spider and bee venoms, toxic polyamines, and toxic peptides or other materials or substances released during bacterial infection (such as bacterial endotoxins and exotoxins), or during protozoal, fungal or viral infection.

- 9 -

Without wishing to be restricted to any particular theory as to mode of action, the dendrimers of the present invention are believed to act as inhibitors of these toxic materials or substances by binding to biological surfaces or receptors and providing multiple sites for interaction with the toxic materials or substances, for example by complexing with these  
5 materials or substances.

The term "inhibition" is used herein in its broadest sense to include either full or partial inhibition or suppression of the toxic effect of the toxic material or substance in a human or non-human animal patient. The term is also used to encompass both prophylactic  
10 and therapeutic treatment.

Thus, in another aspect the present invention provides a pharmaceutical or veterinary composition for prophylactic or therapeutic inhibition of a toxic material or substance in a human or non-human animal patient, which comprises a dendrimer as broadly described  
15 above, in association with at least one pharmaceutically or veterinarily acceptable carrier or diluent.

The formulation of such compositions is well known to persons skilled in this field. Suitable pharmaceutically acceptable carriers and/or diluents include any and all  
20 conventional solvents, dispersion media, fillers, solid carriers, aqueous solutions, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in  
the art, and it is described, by way of example, in *Remington's Pharmaceutical Sciences*, 18th Edition, Mack Publishing Company, Pennsylvania, USA. Except insofar as any  
25 conventional media or agent is incompatible with the active ingredient, use thereof in the pharmaceutical compositions of the present invention is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

It is especially advantageous to formulate compositions in dosage unit form for ease  
30 of administration and uniformity of dosage. Dosage unit form as used herein refers to

- 10 -

physically discrete units suited as unitary dosages for the human subjects to be treated; each unit containing a predetermined quantity of active ingredient calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier and/or diluent. The specifications for the novel dosage unit forms of the invention are dictated by  
5 and directly dependent on (a) the unique characteristics of the active ingredient and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active ingredient for the particular treatment.

In yet another aspect, this invention provides the use of an effective amount of a  
10 dendrimer as broadly described above in the prophylactic or therapeutic treatment of, or in the manufacture of a medicament for prophylactic or therapeutic treatment of a human or non-human animal patient by inhibition of a toxic material or substance.

A variety of administration routes are available. The particular mode selected will  
15 depend, of course, upon the particular condition being treated and the dosage required for therapeutic efficacy. The methods of this invention, generally speaking, may be practised using any mode of administration that is medically acceptable, meaning any mode that produces therapeutic levels of the active component of the invention without causing clinically unacceptable adverse effects. Such modes of administration include oral, rectal,  
20 topical, nasal, inhalation, transdermal or parenteral (e.g. subcutaneous, intramuscular and intravenous) routes. Formulations for oral administration include discrete units such as capsules, tablets, lozenges and the like. Other routes include intrathecal administration  
~~directly into spinal fluid, direct introduction such as by various catheter and balloon~~  
angioplasty devices well known to those of ordinary skill in the art, and intraparenchymal  
25 injection into targeted areas.

The compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Such methods include the step of bringing the active component into association with a carrier which constitutes  
30 one or more accessory ingredients. In general, the compositions are prepared by uniformly

- 11 -

and intimately bringing the active component into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

Compositions of the present invention suitable for oral administration may be  
5 presented as discrete units such as capsules, cachets, tablets or lozenges, each containing a  
predetermined amount of the active component, in liposomes or as a suspension in an  
aqueous liquor or non-aqueous liquid such as a syrup, an elixir, or an emulsion.

Compositions suitable for parenteral administration conveniently comprise a sterile  
10 aqueous preparation of the active component which is preferably isotonic with the blood of  
the recipient. This aqueous preparation may be formulated according to known methods  
using those suitable dispersing or wetting agents and suspending agents. The sterile  
injectable preparation may also be a sterile injectable solution or suspension in a non-toxic  
parenterally-acceptable diluent or solvent, for example as a solution in polyethylene glycol.  
15 Among the acceptable vehicles and solvents that may be employed are water, Ringer's  
solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are  
conventionally employed as a solvent or suspending medium. For this purpose, any bland  
fixed oil may be employed including synthetic mono- or di-glycerides. In addition, fatty  
acids such as oleic acid find use in the preparation of injectables.

20

The active component may also be formulated for delivery in a system designed to  
~~administer the active component intranasally or by inhalation, for example as a finely~~  
dispersed aerosol spray containing the active component.

25 Other delivery systems can include sustained release delivery systems. Preferred  
sustained release delivery systems are those which can provide for release of the active  
component of the invention in sustained release pellets or capsules. Many types of sustained  
release delivery systems are available. These include, but are not limited to: (a) erosional  
systems in which the active component is contained within a matrix, and (b) diffusional  
30 systems in which the active component permeates at a controlled rate through a polymer. In

- 12 -

addition, a pump-based hardware delivery system can be used, some of which are adapted for implantation.

The active component is administered in prophylactically or therapeutically effective  
5 amounts. A prophylactically or therapeutically effective amount means that amount  
necessary at least partly to attain the desired effect, or to delay the onset of, inhibit the  
progression of, or halt altogether, the onset or progression of the particular condition being  
treated. Such amounts will depend, of course, on the particular condition being treated, the  
severity of the condition and individual patient parameters including age, physical condition,  
10 size, weight and concurrent treatment. These factors are well known to those of ordinary  
skill in the art and can be addressed with no more than routine experimentation. It is  
preferred generally that a maximum dose be used, that is, the highest safe dose according to  
sound medical judgement. It will be understood by those of ordinary skill in the art,  
however, that a lower dose or tolerable dose may be administered for medical reasons,  
15 psychological reasons or for virtually any other reasons.

Generally, daily oral doses of active component will be from about 0.01 mg/kg per  
day to 1000 mg/kg per day. Small doses (0.01-1 mg) may be administered initially,  
followed by increasing doses up to about 1000 mg/kg per day. In the event that the response  
20 in a subject is insufficient at such doses, even higher doses (or effective higher doses by a  
different, more localised delivery route) may be employed to the extent patient tolerance  
permits. Multiple doses per day are contemplated to achieve appropriate systemic levels of  
compounds.

---

25 The active component according to the invention may also be presented for use in the  
form of veterinary compositions, which may be prepared, for example, by methods that are  
conventional in the art. Examples of such veterinary compositions include those adapted  
for:

(a) oral administration, external application, for example drenches (e.g. aqueous  
30 or non-aqueous solutions or suspensions); tablets or boluses; powders,

- 13 -

granules or pellets for admixture with feed stuffs; pastes for application to the tongue;

(b) parenteral administration for example by subcutaneous, intramuscular or intravenous injection, e.g. as a sterile solution or suspension; or (when appropriate) by intramammary injection where a suspension or solution is introduced into the udder via the teat;

(c) topical application, e.g. as a cream, ointment or spray applied to the skin; or

(d) intravaginally, e.g. as a pessary, cream or foam.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

Further features of the present invention will be apparent from the following Examples which are included by way of illustration, not limitation of the invention. In the following Examples, PAMAM dendrimers refer to polyamidoamine dendrimers based on an ammonia core as detailed in US Patents Nos. 4,507,466, 4,558,120, 4,568,737 and 4,587,329; PAMAM (EDA) dendrimers refer to polyamidoamine dendrimers based on an ethylene diamine core; and BHAlys<sub>x</sub>lys<sub>y</sub>lys<sub>z</sub> dendrimers refer to polylysine unsymmetrical dendrimers based on a benzhydrylamine core and lysine branching units as described in US Patents Nos. 4,289,872 and 4,410,688. ~~The polyamidoamine dendrimers PAMAM 1.0,~~

PAMAM 2.0, PAMAM 3.0, PAMAM 4.0, PAMAM 5.0 or higher generation, PAMAM 4.0 (EDA), and the polylysine dendrimers BHAlyslys<sub>2</sub>, BHAlyslys<sub>2</sub>lys<sub>4</sub>, BHAlyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub> and BHAlyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub>, BHAlyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub>lys<sub>32</sub>, BHAlyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub>lys<sub>32</sub>lys<sub>64</sub>, or higher generations prepared as described in US Patents Nos. 4,289,872, 4,410,688, 4,507,466, 4,558,120, 4,568,737 and 4,578,239 and International Patent Publications Nos. WO 88/01178, WO 88/01179, WO 88/01180 and WO 95/24221 referred to above.

- 14 -

**EXAMPLE 1**

**Reaction of dendritic polymers with 2-acrylamido-2-methyl propane sulfonic acid to give sulfonic acid terminated dendrimers.**

5

**A PAMAM 1.0**

Solid sodium carbonate (0.13g; 1.0mmol) was added slowly to a stirred solution of 2-acrylamido-2-methyl propane sulfonic acid (0.41g; 2.0mmol) in water (3ml). After the evolution of gas had ceased, the pH of the solution was 8.0. A solution of PAMAM 1.0 (0.12g; 0.33mmol) in water (1ml) was then added to the solution followed by the addition of four drops of a 40% aq. solution of benzyl trimethylammonium hydroxide. The solution was then heated under nitrogen at 60° for three days and then concentrated. The residue was purified by gel filtration (Sephadex G10; water) and then freeze dried to give the sulfonated PAMAM 1.0 dendrimer as an off white solid (0.51g). <sup>1</sup>H and <sup>13</sup>C nmr spectra showed a mixture of dialkylated and monoalkylated PAMAM 1.0 dendrimer ( ca. 70:30). <sup>13</sup>C nmr (D<sub>2</sub>O): δ 31.0, 31.1, 37.1, 37.7, 41.3, 48.6, 51.5, 53.1, 53.4, 55.6, 56.2, 61.2, 61.5, 178.3, 179.0, 179.8.

20

**B PAMAM 2.0**

PAMAM 2.0 was reacted with 2-acrylamido-2-methyl propane sulfonic acid as described above. The crude product was purified by gel filtration (Sephadex G10; water) and then freeze dried to give an off white solid. <sup>1</sup>H and <sup>13</sup>C nmr spectra showed a mixture of dialkylated and monoalkylated PAMAM 2.0 dendrimer ( ca. 65:35). <sup>13</sup>C nmr (D<sub>2</sub>O): δ 31.0, 31.1, 37.1, 37.7, 41.3, 48.7, 51.5, 53.4, 55.6, 56.2, 61.2, 61.5, 178.4, 179.0, 179.1, 179.6.

25

When the above reaction was repeated omitting the benzyltrimethylammonium hydroxide a similar result was obtained.

30



- 15 -

**C PAMAM 3.0 BRI2783**

PAMAM 3.0 was reacted with 2-acrylamido-2-methyl propane sulfonic acid as above except that a slight excess of sodium carbonate was used and the

5 benzyltrimethylammonium hydroxide was omitted.  $^1\text{H}$  and  $^{13}\text{C}$  nmr spectra showed a mixture of dialkylated and monoalkylated PAMAM 3.0 dendrimer (ca. 50:50).  $^{13}\text{C}$  nmr ( $\text{D}_2\text{O}$ ):  $\delta$  31.0, 31.1, 36.9, 37.4, 41.1, 48.6, 51.5, 53.4, 55.7, 56.2, 61.1, 61.5, 178.2, 178.9, 179.0, 179.8.

**10 D PAMAM 4.0 BRI2784**

PAMAM 4.0 was reacted with 2-acrylamido-2-methyl propane sulfonic acid as described for PAMAM 3.0.  $^1\text{H}$  and  $^{13}\text{C}$  nmr spectra showed a mixture of dialkylated and monoalkylated PAMAM 4.0 dendrimer (ca. 35:65).  $^{13}\text{C}$  nmr ( $\text{D}_2\text{O}$ ):  $\delta$  31.0, 15 31.1, 36.9, 37.3, 41.1, 48.5, 51.5, 53.5, 55.7, 56.2, 61.1, 61.5, 178.1, 178.9, 179.0, 179.8.

**EXAMPLE 2****20 Preparation of sodium sulfoacetamide terminated dendrimers.****~~A PAMAM 1.0~~**

25 A solution of 4-nitrophenyl bromoacetate (0.40g; 1.5mmol) in dry DMF (1ml) was added to a stirred solution of PAMAM 1.0 (0.18g; 0.5mmol) in DMF (3ml). The resulting yellow solution was stirred for 20 hours at room temperature, when a ninhydrin test was negative. The solution was concentrated ( $30^\circ/0.1\text{mmHg}$ ) to give a yellow oil. This oil was partitioned between water and chloroform and the aqueous layer separated and washed with chloroform (2X) and finally with ethyl acetate. The 30 aqueous solution was concentrated ( $35^\circ/25\text{mmHg}$ ) to give the bromoacetylated

- 16 -

PAMAM 1.0 dendrimer as a yellow oil (0.36g; 100%).  $^{13}\text{C}$  nmr ( $\text{D}_2\text{O}$ ):  $\delta$  32.8, 33.3, 43.0, 43.5, 54.4, 174.5, 176.4.

5 A solution of sodium sulfite (0.2g; 1.6mmol) in water (1ml) was added to a solution of the bromoacetylated PAMAM 1.0 dendrimer described above (0.36g; 0.5mmol) in water (5ml) and the solution left to stand at room temperature for eleven days. The yellow solution was concentrated to give a yellowish solid (0.60g).  $^{13}\text{C}$  nmr ( $\text{D}_2\text{O}$ ):  $\delta$  34.4, 43.1, 43.4, 54.0, 61.7, 171.3, 177.2.

10 The above reaction sequence could be carried out without isolating the bromoacetylated dendrimer by simply adding the sodium sulfite solution to the crude aqueous extract obtained from the first reaction.

## B PAMAM 2.0

15

### Method 1:

A solution of 4-nitrophenyl bromoacetate (0.18g; 0.7mmol) in dry DMF (1ml) was added to a stirred solution of PAMAM 2.0 (0.10g; 0.1mmol) in DMF (3ml). The resulting yellow solution was stirred for 20 hours at room temperature, when a ninhydrin test was negative. The solution was then added with swirling to water (150ml) and the mixture extracted with chloroform (3X) and ethyl acetate. A solution of sodium sulfite (0.1g; 0.8mmol) in water (1ml) was added to the crude bromoacetylated dendrimer solution and the mixture allowed to stand for three days at room temperature. The yellowish solution was then concentrated to give a yellow solid residue, which was purified by gel filtration (Sephadex LH20; water) to give the sodium sulfoacetamide terminated PAMAM 2.0 dendrimer (103mg).  $^{13}\text{C}$  nmr ( $\text{D}_2\text{O}$ ):  $\delta$  33.0, 35.7, 36.0, 37.7, 40.3, 43.0, 43.2, 53.4, 53.7, 56.0, 61.6, 171.2, 174.6, 178.5.

30

- 17 -

## Method 2:

5 Solid succinimidyl acetylthioacetate (67mg; 0.33mmol) was added to a solution of PAMAM 2.0 (52mg; 0.05mmol) in dry DMF (2ml) and the resulting solution stirred at room temperature for two days. The mixture was then concentrated ( $30^{\circ}/10^{-3}$  mmHg) to give an oily residue. The residue was partitioned between water and chloroform, and the water layer separated and concentrated to give a viscous oil (117mg).  $^1\text{H}$  and  $^{13}\text{C}$  nmr showed the oil to be a mixture of the acylated dendrimer and N-hydroxy succinimide. Gel filtration (Sephadex G10; water) provide a pure sample of the acetylthioacetamide terminated PAMAM 2.0 dendrimer (29mg).  $^{13}\text{C}$  nmr ( $\text{D}_2\text{O}$ ):  $\delta$  34.0, 34.2, 37.3, 43.0, 43.1, 43.3, 53.5, 54.0, 56.3, 175.4, 177.2, 177.5.

15 A solution of the above functionalised dendrimer in 40% aqueous formic acid (7ml) was then added to an ice cold freshly prepared solution of performic acid (1.6mmol) in formic acid (2ml). The mixture was stirred for one hour at  $0^{\circ}$  and then for twenty hours at room temperature. A small amount of activated charcoal was then added to decompose any excess peracid, the mixture stirred for 30 minutes then filtered and concentrated to give a viscous oil.

20 The crude product was dissolved in water, the pH adjusted to 9.0 with aqueous sodium bicarbonate and the material desalted by passage through a column of Sephadex G10. A white solid (20mg; ) was obtained after lyophilisation which was spectroscopically essentially the same as the material obtained by method 1.  $^{13}\text{C}$  nmr ( $\text{D}_2\text{O}$ ):  $\delta$  33.0, 38.7, 42.9, 43.0, 43.1, 53.9, 54.3, 56.5, 61.6, 171.2, 176.4, 177.0.

**EXAMPLE 3****Preparation of sodium sulfosuccinamic acid terminated dendrimers**

- 18 -

## A PAMAM 1.0

Solid maleic anhydride (0.11g; 1.1mmol) was added to a stirred solution of PAMAM 1.0 (0.12g; 0.33mmol) in dry DMF (3ml). The mixture became a little warm and brownish as the anhydride dissolved and the resulting solution was stirred overnight at room temperature. The solution was then concentrated ( $30^{\circ}/10^{-4}$  mmHg) to give a viscous oil.  $^1\text{H}$  and  $^{13}\text{C}$  nmr ( $\text{D}_2\text{O}$ ) showed complete conversion of the PAMAM 1.0 to the trisamide together with some maleic acid.  $^{13}\text{C}$  nmr ( $\text{D}_2\text{O}$ ):  $\delta$  33.1, 42.8, 43.1, 54.3, 135.0, 137.1, 169.1, 171.9, 173.3.

The crude trisamide was then dissolved in water (4ml) and solid sodium sulfite (0.20g; 1.6mmol) added. The resulting solution was allowed to stand at room temperature for four days and then concentrated.  $^1\text{H}$  and  $^{13}\text{C}$  nmr ( $\text{D}_2\text{O}$ ) showed a 1:1 mixture of the regioisomeric sodium sulfosuccinamic acid terminated PAMAM 1.0 dendrimers together with some sulfosuccinic acid. The crude product was purified by gel filtration (Sephadex G10; water) to afford a sample of the sodium sulfosuccinamic acid terminated PAMAM 1.0 dendrimers (107mg).  $^{13}\text{C}$  nmr ( $\text{D}_2\text{O}$ ):  $\delta$  33.3, 39.6, 40.0, 42.9, 43.1, 54.0, 67.9, 69.4, 173.8, 176.3, 177.6, 181.8.

## 20 B PAMAM 2.0

A mixture of the regioisomeric sodium sulfosuccinamic acid terminated PAMAM 2.0 dendrimers was prepared as described above.  $^{13}\text{C}$  nmr PAMAM 2.0 maleamic acid derivative ( $\text{D}_2\text{O}$ ):  $\delta$  32.8, 33.0, 38.7, 42.9, 53.8, 54.3, 56.5, 135.2, 136.8, 169.2, 171.9, 173.5, 174.6.  $^{13}\text{C}$  nmr PAMAM 2.0 sodium sulfosuccinamic acid derivatives ( $\text{D}_2\text{O}$ ):  $\delta$  37.0, 40.1, 41.1, 43.0, 43.2, 43.9, 53.0, 53.3, 55.5, 68.0, 69.4, 173.8, 177.6, 179.1, 179.5, 179.8, 182.3.

- 19 -

**C PAMAM 4.0 BRI6038**

Solid maleic anhydride (60mg; 0.6mmol) was added to a stirred solution of PAMAM 4.0 (51mg; 0.01mmol) in dry DMF (2ml). The mixture initially became cloudy but soon gave a clear solution which was stirred overnight at room temperature. The solution was then concentrated (35°/10<sup>-4</sup> mmHg) to give a viscous oil. <sup>1</sup>H and <sup>13</sup>C nmr (D<sub>2</sub>O) showed complete conversion of the PAMAM 4.0 to the polyamide together with some maleic acid. The crude polyamide was then dissolved in water (2ml) and a solution of sodium sulfite (126mg; 1.0mmol) in water (2ml) added. The resulting solution was allowed to stand at room temperature for two days and then concentrated. <sup>1</sup>H and <sup>13</sup>C nmr (D<sub>2</sub>O) showed a mixture of the regioisomeric sodium sulfosuccinamic acid terminated PAMAM 4.0 dendrimers together with some sulfosuccinic acid. The crude product was purified by gel filtration (Sephadex LH20; water) to afford a sample of PAMAM 4.0 terminated with 24 regioisomeric sulfosuccinamic acid groups (90mg). <sup>1</sup>H nmr (D<sub>2</sub>O): δ 2.4-2.6; 2.7-3.1; 3.2-3.4; 3.9-4.0. <sup>13</sup>C nmr (D<sub>2</sub>O): δ 36.2; 39.8; 40.5; 43.0; 43.2; 53.5; 55.8; 68.1; 69.5; 173.8; 177.4; 177.6; 178.7; 182.3.

**EXAMPLE 4****Preparation of sodium N-(2-sulfoethyl)succinamide terminated dendrimers****a Preparation of tetrabutylammonium N-(2-sulfoethyl)succinamic acid**

Solid succinic anhydride (0.5g; 5.0mmol) was added to a stirred solution of tetrabutylammonium 2-aminoethylsulfonic acid (1.83g; 5.0mmol) in dry dichloromethane (30ml). The succinic anhydride slowly dissolved and the resulting cloudy solution was stirred overnight at room temperature. The mixture was filtered and the filtrate concentrated to give a viscous oil (2.41g). <sup>13</sup>C nmr showed complete

- 20 -

conversion to the desired monoamide together with a small amount of succinic acid. Repeated precipitation of the product by dropwise addition of a dichloromethane solution to a large excess of diethyl ether gave tetrabutylammonium N-(2-sulfoethyl)succinamic acid as a white solid (1.762g; 76% ), mp 125-127°C. <sup>1</sup>H nmr (CDCl<sub>3</sub>): δ 0.86 (t, 12h, 4xCH<sub>3</sub>), 1.28 (m, 8H, 4xCH<sub>2</sub>), 1.50 (m, 8H, 4xCH<sub>2</sub>), 2.33 (m, 2H, CH<sub>2</sub>COOH), 2.44 (m, 2H, CH<sub>2</sub>CONH), 2.76 (m, 2H, CH<sub>2</sub>NHCO), 3.12 (m, 8H, 4xCH<sub>2</sub>N), 3.50 (m, 2H, CH<sub>2</sub>SO<sub>3</sub><sup>-</sup>), 7.53 (br t, 1H, NH). <sup>13</sup>C nmr (CDCl<sub>3</sub>): δ 13.5, 19.5, 23.8, 30.1, 30.9, 35.6, 50.0, 58.5, 172.0, 174.1.

10 b Preparation of tetrabutylammonium 4-nitrophenyl N-(2-sulfoethyl)succinamate

A solution of dicyclohexylcarbodiimide (45mg; 0.22mmol) in dry dichloromethane (1ml) was added to a stirred solution of tetrabutylammonium N-(2-sulfoethyl)succinamic acid (94mg; 0.20mmol) in dichloromethane (2ml), and the mixture stirred overnight at room temperature. The resulting suspension was filtered and the filtrate concentrated to give the crude active ester, which was used without further purification.

A Preparation of sodium N-(2-sulfoethyl)succinamide terminated PAMAM dendrimers

20

PAMAM 4.0 BRI2786

A solution of the crude tetrabutylammonium 4-nitrophenyl N-(2-sulfoethyl)succinamate (0.30mmol) in dry DMF (1ml) was added to a stirred solution of PAMAM 4.0 (51.5mg; 0.01mmol) dissolved in 50% aqueous DMF (3ml) and the resulting yellow solution stirred overnight at room temperature. The mixture was then concentrated (35°/10<sup>-5</sup> mmHg) and the yellow residue partitioned between water and chloroform. The water layer was separated, washed with chloroform (2X) and ethyl acetate, and then concentrated to give a yellow oil (134mg). The crude product was converted to the sodium salt by passage through a column of Amberlite

- 21 -

IR 120(Na) to yield 85mg of material. This material was further purified by gel filtration (Sephadex LH20; water) to give the sodium N-(2-sulfoethyl)succinamide terminated PAMAM 4.0 dendrimer (45mg).  $^{13}\text{C}$  nmr ( $\text{D}_2\text{O}$ ):  $\delta$  33.2, 33.6, 35.5, 39.0, 39.5, 42.8, 43.2, 53.8, 54.1, 54.4, 56.6, 176.5, 176.9, 177.2, 178.9, 179.4.

5

The corresponding PAMAM-1.0 and PAMAM-3.0 (**BRI2785**) dendrimers terminated with sodium N-(2-sulfoethyl)succinamide groups were similarly prepared.

$^{13}\text{C}$  nmr PAMAM 3.0 derivative ( $\text{D}_2\text{O}$ ):  $\delta$  33.4, 35.5, 39.0, 39.5, 42.9, 43.2, 53.8, 54.1, 54.3, 56.5, 176.4, 176.9, 177.4, 178.9, 179.4.

10

$^{13}\text{C}$  nmr PAMAM 1.0 derivative ( $\text{D}_2\text{O}$ ):  $\delta$  34.9, 35.5, 39.5, 42.9, 43.1, 53.7, 54.1, 179.0, 179.1, 179.3.

15 B

Preparation of sodium N-(2-sulfoethyl)succinamide terminated polylysine dendrimers

BHAlyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub> **BRI2789**

20

Trifluoroacetic acid (1ml) was added to a suspension of BHAlyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>DBL<sub>16</sub> (36.5mg; 5.0 $\mu$ mol) in dry dichloromethane (1ml) and the resulting solution stirred at room temperature under nitrogen for two hours and then concentrated. The residue

was dissolved in dry DMSO (2ml) and the pH adjusted to 8.5 with triethylamine. A solution of the crude tetrabutylammonium 4-nitrophenyl N-(2-

25

sulfoethyl)succinamate (ca. 0.2mmol) in DMSO (1ml) was then added dropwise and the mixture stirred overnight at room temperature. The yellow solution was then concentrated (50 $^{\circ}$ /10 $^{-5}$  mmHg) and the yellow residue partitioned between water and chloroform. The aqueous layer was separated, washed with chloroform (3X) and ethyl acetate, and then concentrated to give an oil (99mg). The crude product was

30

converted to the sodium salt by passage through a column of Amberlite IR 120(Na) to yield 81mg of material. This material was further purified by gel filtration

- 22 -

(Sephadex LH20; water) to give the sodium N-(2-sulfoethyl)succinamide terminated BHAlyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub> dendrimer (39mg). <sup>13</sup>C nmr (D<sub>2</sub>O): δ 27.0, 32.3, 35.2, 35.3, 35.6, 35.7, 39.5, 43.5, 54.1, 58.5, 131.5, 132.0, 133.3, 145.1, 177.8, 178.0, 178.4, 178.8, 178.9, 179.2, 179.7, 179.8.

5

The corresponding BHAlyslys<sub>2</sub>, BHAlyslys<sub>2</sub>lys<sub>4</sub> (**BRI2787**) and BHAlyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub> (**BRI2788**) terminated with sodium N-(2-sulfoethyl)succinamide groups were similarly prepared.

10

<sup>13</sup>C nmr BHAlyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub> derivative (D<sub>2</sub>O): δ 26.9, 32.3, 35.1, 35.3, 35.6, 35.7, 39.5, 43.5, 54.1, 58.5, 131.6, 131.9, 132.2, 132.3, 133.2, 133.3, 145.0, 145.2, 177.2, 177.8, 177.9, 178.0, 178.2, 178.3, 178.6, 178.7, 178.8, 178.9, 179.2, 179.3, 179.7, 179.8.

15

<sup>13</sup>C nmr BHAlyslys<sub>2</sub>lys<sub>4</sub> derivative (D<sub>2</sub>O): δ 26.9, 32.3, 35.1, 35.4, 35.7, 35.8, 39.5, 43.5, 54.1, 58.5, 61.8, 131.7, 132.0, 132.2, 132.3, 133.2, 133.3, 145.0, 145.1, 177.3, 178.0, 178.3, 178.4, 178.7, 178.9, 179.0, 179.3, 179.7, 179.8.

<sup>13</sup>C nmr BHAlyslys<sub>2</sub> derivative (D<sub>2</sub>O): δ 26.9, 27.1, 32.2, 32.3, 34.7, 34.8, 35.1, 35.3, 35.6, 35.7, 39.5, 43.4, 54.1, 58.6, 61.8, 131.7, 131.9, 132.2, 132.3, 133.3, 144.9, 145.0, 177.7, 178.4, 178.8, 179.0, 179.3, 180.0.

20

## EXAMPLE 5

### Preparation of sodium 4-sulfophenylthiourea terminated dendrimers

25

#### A PAMAM 4.0 **BRI2791**

30

Solid sodium 4-sulfophenylisothiocyanate monohydrate (500mg; 1.96mmol) was added to a solution of PAMAM 4.0 (300mg; 0.0582mmol) in water (10ml) and the resulting solution heated under nitrogen at 53° for two hours and then cooled. The



- 23 -

solution was concentrated and the yellow solid residue purified by gel filtration (Sephadex LH20; water). The pure fractions were combined and freeze dried to give the sodium 4-sulfophenylthiourea terminated PAMAM 4.0 dendrimer as a fluffy white solid (370mg).  $^1\text{H}$  nmr ( $\text{D}_2\text{O}$ ) :  $\delta$  2.28; 2.52; 2.69; 3.15; 3.27; 3.60; 7.32 (d,  $J=9\text{Hz}$ ); 7.72 (d,  $J=9\text{Hz}$ ).  $^{13}\text{C}$  nmr ( $\text{D}_2\text{O}$ ) :  $\delta$  36.9; 41.1; 43.1; 48.3; 53.6; 55.8; 129.0; 131.1; 144.4; 178.5; 179.1; 184.4.

The corresponding PAMAM 1.0, PAMAM 2.0 (**BRI2790**), PAMAM 3.0, and PAMAM 5.0 (**BRI2991**) dendrimers terminated with 3, 6, 12, and 48 sodium 4-sulfophenylthiourea groups respectively were similarly prepared.

#### B PAMAM 4.0 (EDA) **BRI6045**

Solid sodium 4-sulfophenylisothiocyanate monohydrate (130mg; 0.5mmol) was added to a solution of PAMAM 4.0 (EDA) (69mg; 0.01mmol) in water (4ml) and the resulting solution heated under nitrogen at  $53^\circ$  for two hours and then cooled. The solution was concentrated and the solid residue purified by gel filtration (Sephadex LH20; water). The pure fractions were combined and freeze dried to give PAMAM 4.0 terminated with 32 sodium 4-sulfophenylthiourea groups as a fluffy white solid (136mg).  $^1\text{H}$  nmr ( $\text{D}_2\text{O}$ ) :  $\delta$  2.30; 2.50; 2.70; 3.18; 3.62; 7.35 (d,  $J=9\text{Hz}$ ); 7.72 (d,  $J=9\text{Hz}$ ).  $^{13}\text{C}$  nmr ( $\text{D}_2\text{O}$ ) :  $\delta$  36.8; 41.0; 43.1; 48.4; 53.6; 55.7; 128.9; 131.0; 144.3; 178.5; 179.0; 184.5.

#### C BHAllyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub> **BRI2792**

Trifluoroacetic acid (4ml) was added to a suspension of BHAllyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>DBL<sub>16</sub> (0.73g; 0.1mmol) in dry dichloromethane (4ml) under nitrogen. A vigorous evolution of gas was observed for a short time and the resulting solution was stirred at room temperature for two hours and then concentrated. The residual syrup was dissolved in water (5ml), the solution passed through a column of Amberlite IRA-401(OH) and the filtrate concentrated to give BHAllyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub> as a viscous oil (0.49g). The

- 24 -

oil was redissolved in water (5ml) and N,N-dimethyl-N-allylamine buffer (pH 9.5; 3ml) added. Solid sodium 4-sulfophenylisothiocyanate monohydrate (1.30g; 5.1mmol) was then added and the resulting solution heated under nitrogen at 53° for two hours and then cooled. The solution was concentrated and the brownish solid residue purified by gel filtration (Sephadex LH20; water). The pure fractions were combined, passed through a column of Amberlite IR 120(Na) and freeze dried to give the sodium 4-sulfophenylthiourea terminated BHAlsyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub> dendrimer as a fluffy white solid (374mg). <sup>1</sup>H nmr (D<sub>2</sub>O) : δ 1.40; 1.72; 3.08; 3.42; 4.24; 4.60; 7.30; 7.40 (d, J=9Hz); 7.78 (d, J=9Hz). <sup>13</sup>C nmr (D<sub>2</sub>O) : δ 27.3; 32.5; 35.9; 43.7; 48.9; 58.6; 63.3; 128.8; 131.0; 143.7; 144.7; 145.1; 177.7; 178.1; 183.8; 185.2.

The corresponding BHAlsyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>, BHAlsyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub>lys<sub>32</sub> (**BRI2992**), and BHAlsyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub>lys<sub>32</sub>lys<sub>64</sub> (**BRI2993**) dendrimers terminated with 16, 64, and 128 sodium 4-sulfophenylthiourea groups respectively were similarly prepared.

## EXAMPLE 6

### 20 Preparation of sodium 3,6-disulfonaphthylthiourea terminated dendrimers

#### A PAMAM 4.0 **BRI2923**

Solid sodium 3,6-disulfonaphthylisothiocyanate (160mg; 0.41mmol) was added to a solution of PAMAM 4.0 (51mg; 0.01mmol) in water (3ml) and the resulting solution heated under nitrogen at 53° for two hours and then cooled. The solution was concentrated and the brown solid residue purified by gel filtration (Sephadex LH20; water). The pure fractions were combined and concentrated to give the sodium 3,6-disulfonaphthylthiourea terminated PAMAM 4.0 dendrimer as a brownish solid (73mg). <sup>1</sup>H nmr (D<sub>2</sub>O) : δ 2.30; 2.60; 2.74; 3.20; 3.57; 7.75; 7.86; 8.28. <sup>13</sup>C nmr

- 25 -

(D<sub>2</sub>O) :  $\delta$  35.0; 39.9; 43.1; 48.1; 53.8; 56.1; 128.4; 128.6; 129.3; 131.0; 131.3; 136.0; 136.8; 138.2; 145.5; 146.0; 177.2; 177.8; 185.5.

The corresponding PAMAM 2.0 dendrimer terminated with sodium 3,6-disulfonaphthylthiourea groups was similarly prepared.

## B PAMAM 4.0 (EDA) **BRI6046**

Solid sodium 3,6-disulfonaphthylisothiocyanate (220mg; 0.57mmol) was added to a solution of PAMAM 4.0 (EDA) (74mg; 0.01mmol) in water (4ml) and the resulting solution heated under nitrogen at 53° for two hours and then cooled. The solution was concentrated and the brownish solid residue purified by gel filtration (Sephadex LH20; water). The pure fractions were combined and concentrated to give PAMAM 4.0 terminated with 32 sodium 3,6-disulfonaphthylthiourea groups as a tan solid (148mg). <sup>1</sup>H nmr (D<sub>2</sub>O) :  $\delta$  2.30; 2.80; 3.20; 3.54; 7.74; 7.85; 8.25. <sup>13</sup>C nmr (D<sub>2</sub>O) :  $\delta$  36.0; 40.8; 43.1; 48.3; 53.6; 55.9; 128.5; 129.4; 131.0; 131.3; 136.0; 136.8; 138.3; 145.5; 146.0; 178.2; 185.6.

## 20 C BHAlyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub> **BRI2999**

Trifluoroacetic acid (2ml) was added to a suspension of BHAlyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>DBL<sub>16</sub> (73mg; 0.01mmol) in dry dichloromethane (2ml) under nitrogen. A vigorous evolution of gas was observed for a short time and the resulting solution was stirred at room temperature for two hours and then concentrated. The residual syrup was dissolved in water (5ml), the solution passed through a column of Amberlite IRA-401(OH) and the filtrate concentrated to give BHAlyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub> as a viscous oil. The oil was redissolved in water (5ml) and N,N-dimethyl-N-allylamine buffer (pH 9.5; 3ml) added. Solid sodium 3,6-disulfonaphthylisothiocyanate (234mg; 0.60mmol) was then added and the resulting solution heated under nitrogen at 53°

- 26 -

for two hours and then cooled. The solution was concentrated and the brownish solid residue purified by gel filtration (Sephadex LH20; water). The pure fractions were combined, passed through a column of Amberlite IR 120(Na) and freeze dried to give BHAlyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub> terminated with 32 sodium 3,6-disulfonaphthylthiourea groups as a fluffy off-white solid (119mg). <sup>1</sup>H nmr (D<sub>2</sub>O) : δ 1.0-2.0; 3.18; 3.43; 4.31; 7.22; 7.80; 7.89; 8.25. <sup>13</sup>C nmr (D<sub>2</sub>O) : δ 27.2; 32.4; 35.3; 43.7; 49.0; 58.5; 63.6; 128.4; 129.1; 131.4; 136.1; 136.6; 138.6; 139.0; 145.1; 145.6; 178.4; 184.8; 186.7.

10

**EXAMPLE 7****Preparation of sodium 4-sulfonaphthylthiourea terminated dendrimers****PAMAM 4.0 BRI2997**

15

Solid sodium 4-sulfonaphthylisothiocyanate (180mg; 0.5mmol) was added to a solution of PAMAM 4.0 (51mg; 0.01mmol) in water (5ml) and the mixture heated under nitrogen at 53° for two hours and then cooled. The water was distilled under reduced pressure from the resulting suspension and the off white solid residue purified by gel filtration (Sephadex  
20 LH20; water). The pure fractions were combined and freeze dried to give the sodium 4-sulfonaphthylthiourea terminated PAMAM 4.0 dendrimer as a fluffy white solid (60mg). <sup>1</sup>H nmr (D<sub>2</sub>O) : δ 2.20; 2.60; 3.14; 3.48; 7.23; 7.47; 7.56; 7.77; 7.93 (d, J=6Hz); 8.56 (d, J=6Hz). <sup>13</sup>C nmr (D<sub>2</sub>O) : δ 35.8; 40.5; 43.1; 48.4; 53.6; 55.9; 127.6; 128.6; 130.3; 131.9; 132.5; 133.5; 134.7; 140.5; 142.7; 177.8; 178.0; 185.4.

25

- 27 -

**EXAMPLE 8****Preparation of sodium 3,5-disulfophenylthiourea terminated dendrimers****5 PAMAM 4.0 BRI6039**

Solid sodium 3,5-disulfophenylisothiocyanate (110mg; 0.32mmol) was added to a solution of PAMAM 4.0 (63mg; 0.012mmol) in water (3ml) and the resulting solution heated under nitrogen at 53° for two hours and then cooled. The solution was concentrated and the brownish solid residue purified by gel filtration (Sephadex G25; water). The pure fractions were combined and concentrated to give PAMAM 4.0 terminated with 24 sodium 3,5-disulfophenylthiourea groups as an off-white solid (110mg). <sup>1</sup>H nmr (D<sub>2</sub>O) : δ 2.53; 3.08; 3.36; 3.66; 7.90; 7.95. <sup>13</sup>C nmr (D<sub>2</sub>O) : δ 34.8; 41.0; 43.1; 48.0; 53.7; 56.2; 124.1; 128.6; 143.5; 148.8; 177.6; 185.0.

15

**EXAMPLE 9****Preparation of sodium 3, 6, 8-trisulfonaphthylthiourea terminated dendrimers****20 PAMAM 4.0 BRI2998**

Solid sodium 3, 6, 8-trisulfonaphthylisothiocyanate (250mg; 0.5mmol) was added to a  
solution of PAMAM 4.0 (51mg; 0.01mmol) and N,N-dimethyl-N-allylamine buffer (pH 9.5; 1ml) in water (2ml) and the mixture heated under nitrogen at 53° for two hours and then cooled. The mixture was concentrated under reduced pressure to give an orange solid. The residual solid was dissolved in water (2ml) and passed through a short column of Amberlite IR-120(Na). The filtrate was then concentrated and the residue purified by gel filtration (Sephadex LH20; water). The pure fractions were combined and freeze dried to give the sodium 3, 6, 8-trisulfonaphthylthiourea terminated PAMAM 4.0 dendrimer as an off-white solid (102mg). <sup>1</sup>H nmr (D<sub>2</sub>O) : δ 2.65; 3.02; 3.30; 3.66; 8.05; 8.42; 8.59; 8.67. <sup>13</sup>C nmr

30

- 28 -

(D<sub>2</sub>O) :  $\delta$  33.2; 38.7; 43.2; 43.7; 47.8; 54.0; 54.3; 56.7; 131.0; 131.3; 131.9; 135.9; 138.0; 139.6; 143.8; 144.1; 145.6; 176.2; 176.5; 186.0.

### EXAMPLE 10

5

#### Preparation of sodium 4-(sulfomethyl)benzamide terminated dendrimers

##### PAMAM 4.0 BRI6040

- 10 Solid 4-nitrophenyl 4-(chloromethyl)benzoate (200mg; 0.68mmol) was added to a stirred solution of PAMAM 4.0 (70mg; 0.014mmol) in dry DMSO (4ml) and the resulting yellow solution stirred at room temperature for two hours. The solution was then concentrated ( $10^{-4}$  mmHg; 40<sup>o</sup>) and the residue extracted with a mixture of water and dichloromethane (1:1). The remaining solid material was dissolved in DMSO (5ml) and a solution of sodium sulfite
- 15 (130mg; 1mmol) in water (3ml) added. The slightly cloudy mixture that resulted was left to stand for four days, after which time the addition of more water (2ml) resulted in the formation of a clear homogeneous yellow solution. The solution was then concentrated, first at 25mmHg and 40<sup>o</sup> then at  $10^{-4}$  mmHg and 50<sup>o</sup> to give the crude product. The crude product was purified by gel filtration (Sephadex G25; water) to give PAMAM 4.0
- 20 terminated with 24 sodium 4-(sulfomethyl)benzamide groups (24mg). <sup>1</sup>H nmr (D<sub>2</sub>O) :  $\delta$  2.25; 2.66; 3.08; 3.20; 3.33; 3.38; 4.01; 7.40 (br d); 7.62 (br d). <sup>13</sup>C nmr (D<sub>2</sub>O) :  $\delta$  36.7; 40.9; 43.0; 43.6; 53.5; 55.5; 61.0; 131.6; 135.0; 137.2; 140.4; 174.5; 178.6; 179.2.

**EXAMPLE 11****Preparation of 4-sulfobenzamide terminated dendrimers****5 PAMAM 4.0 (EDA) BRI6116**

Solid potassium N-hydroxysuccinimidyl 4-sulfobenzoate (100mg; 0.3mmol) was added to a solution of PAMAM 4.0 (EDA) (35mg; 0.005mmol) in 0.1M pH 8.5 borate buffer (5ml) and the solution stirred at room temperature for two hours. The resulting milky solution at this stage had a pH of 4.5. 1M Sodium carbonate solution (1ml) was then added to give a clear solution which was concentrated to give the crude product as a white solid. The crude product was purified by gel filtration (Sephadex G25; water) to give PAMAM 4.0 (EDA) terminated with 32 sodium 4-sulfobenzamide groups (47mg).  $^1\text{H}$  nmr ( $\text{D}_2\text{O}$ ) :  $\delta$  2.25; 2.42; 2.63; 3.05; 3.18; 3.31; 3.38; 7.72 (d, J=8Hz); 7.78 (d, J=8Hz).  $^{13}\text{C}$  nmr ( $\text{D}_2\text{O}$ ) :  $\delta$  36.0; 40.4; 43.0; 43.7; 53.7; 55.8; 130.2; 132.2; 140.4; 150.1; 173.6; 178.0; 178.5.

**EXAMPLE 12****Preparation of Sodium N-(4-sulfophenyl)propanamide terminated dendrimers**

20

**PAMAM 4.0 (EDA) BRI6117**

Solid sodium N-(4-sulfophenyl)acrylamide (250mg; 1mmol) and solid sodium carbonate (106mg; 1mmol) were added successively to a stirred solution of PAMAM 4.0 (EDA) (78mg; 0.011mmol) in water (4ml). The resulting solution was stirred under nitrogen for four days and then freeze dried to give a fluffy white solid. The crude product was purified by gel filtration (Sephadex LH20; water) to give PAMAM 4.0 (EDA) terminated with 64 sodium N-(4-sulfophenyl)propanamide groups (206mg).  $^{13}\text{C}$  nmr showed a faint trace of

- 30 -

what was taken to be mono alkylated terminal amino groups.  $^1\text{H}$  nmr ( $\text{D}_2\text{O}$ ) :  $\delta$  2.10; 2.48; 2.58; 2.79; 3.20; 7.42 (d,  $J=7\text{Hz}$ ); 7.65 (d,  $J=7\text{Hz}$ ).  $^{13}\text{C}$  nmr ( $\text{D}_2\text{O}$ ) :  $\delta$  36.5; 37.9; 41.1; 53.4; 55.6; 124.8; 130.9; 143.0; 144.2; 177.4; 178.5.

5

**EXAMPLE 13****Preparation of Sodium 4-sulfohenylurea terminated dendrimers**PAMAM 4.0 (EDA) **BRI6115**

10

A solution of sodium sulfanilic acid (195mg; 1mmol) in dry DMSO (3ml) was added dropwise to a solution of N,N'- disuccinimidyl carbonate (530mg; 2mmol) in dry DMSO (4ml) and the resulting brownish solution stirred at room temperature for 20 hours. A solution of PAMAM 4.0 (EDA) (75mg; 0.011mmol) in dry DMSO (1ml) added and the solution stirred for a further 18 hours. The solution was then concentrated under high vacuum ( $10^{-5}$  mmHg;  $35^\circ$ ) to give a yellowish semi-solid. The crude product was dissolved in DMSO (4ml) and the solution added to 200ml of well stirred ethyl acetate. The precipitated white solid was collected by filtration and washed with ethyl acetate (2X) and ether (2X), then dried to give a white powder (275mg). This material was further purified by gel filtration (Sephadex LH20; water) to give PAMAM 4.0 (EDA) terminated with 32 sodium 4-sulfohenylurea groups (106mg).  $^1\text{H}$  nmr ( $\text{D}_2\text{O}$ ) :  $\delta$  2.31; 2.55; 2.75; 3.19; 7.32 (d,  $J=9\text{Hz}$ ); 7.63 (d,  $J=9\text{Hz}$ ).  $^{13}\text{C}$  nmr ( $\text{D}_2\text{O}$ ) :  $\delta$  36.3; 40.7; 43.3; 43.8; 53.7; 55.7; 123.3; 130.9; 140.9; 146.0; 161.4; 178.2; 178.6.



- 31 -

**EXAMPLE 14****Preparation of N,N,N-trimethylglycinamide chloride terminated dendrimers****5 BHAllyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub> BRI2922**

Trifluoroacetic acid (4ml) was added to a suspension of BHAllyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>DBL<sub>16</sub> (220mg; 30 $\mu$ mol) in dry dichloromethane (2ml) and the resulting solution stirred at room temperature under nitrogen for two hours and then concentrated. The residue was dissolved in dry

10 DMSO (5ml) and the pH adjusted to 8.5 with triethylamine. Solid 4-nitrophenyl N,N,N-trimethylglycinate chloride (0.50g; 1.8mmol) was then added and the mixture stirred overnight at room temperature. The cloudy solution was then concentrated (50°/10<sup>-5</sup> mmHg) and the residue partitioned between water and dichloromethane. The aqueous layer was separated, washed with dichloromethane (3X) and ethyl acetate, and then concentrated to

15 give an oil (1.128g). The crude product was purified by gel filtration (Sephadex LH20; water) to give the N,N,N-trimethylglycinamide terminated BHAllyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub> dendrimer (116mg). <sup>13</sup>C nmr (D<sub>2</sub>O):  $\delta$  25.5, 30.5, 30.8, 33.4, 42.1, 56.5, 57.1, 67.5, 68.1, 166.7, 167.0, 167.1, 176.0, 176.2.

20

**EXAMPLE 15****~~Preparation of 4-Trimethylammoniumbenzamide terminated dendrimers~~****PAMAM 4.0 BRI6043**

25

1,1'-Carbonyldiimidazole (85mg; 0.52mmol) was added to a solution of 4-trimethylammoniumbenzoic acid iodide (154mg; 0.5mmol) in dry DMF (4ml) and the mixture stirred at room temperature under argon for two hours. During this time a white solid separated from the solution. A solution of PAMAM 4.0 (58mg; 0.011mmol) in dry

30 DMF (2ml) was then added and the mixture stirred overnight at room temperature. After this

time most of the precipitate had dissolved and a ninhydrin test of the solution was negative. The mixture was concentrated ( $10^{-4}$  mmHg;  $30^{\circ}$ ) to give a white solid residue. The crude product was purified by gel filtration (Sephadex LH20; 10% AcOH) to give PAMAM 4.0 terminated with 24 4-trimethylammoniumbenzamide groups as the acetic acid salt (89mg).  
5  $^1\text{H}$  nmr ( $\text{D}_2\text{O}$ ) :  $\delta$  1.96; 2.65-2.85; 3.25-3.55; 3.64; 7.92.  $^{13}\text{C}$  nmr ( $\text{D}_2\text{O}$ ) :  $\delta$  25.8; 33.1; 33.5; 38.7; 43.1; 43.5; 53.5; 54.1; 56.4; 61.2; 124.8; 133.6; 139.9; 153.2; 173.2; 176.3; 176.8; 182.6.

The corresponding PAMAM 2.0 dendrimer terminated with 6 4-trimethylammonium  
10 benzamide groups was similarly prepared.

## EXAMPLE 16

### Preparation of 4-(Trimethylammoniummethyl)benzamide terminated 15 dendrimers

#### PAMAM 4.0 BRI6044

Solid 4-nitrophenyl 4-(chloromethyl)benzoate (150mg; 0.5mmol) was added to a  
20 stirred solution of PAMAM 4.0 (52mg; 0.01mmol) in dry DMSO (3ml). The resulting yellow solution was stirred at room temperature for 20 hours, when a ninhydrin test was negative (pH ca.8.5). The solution was then concentrated ( $10^{-5}$  mmHg;  $40^{\circ}$ ) and the residue shaken with a mixture of water and dichloromethane (1:1). The insoluble gel-like material was collected by filtration, washed with water  
25 (2X) and dichloromethane (2X), and then air dried. The crude 4-(chloromethyl)-benzamide terminated dendrimer was dissolved in 25% aq. trimethylamine (20ml) and the yellow solution left to stand overnight. The solution was then concentrated, the residue dissolved in water (5ml) and the solution passed through a column of Amberlite IRA-401 (OH). The colourless filtrate was concentrated to give a viscous  
30 oil which was purified by gel filtration (Sephadex G10; 10% AcOH) to give

- 33 -

PAMAM 4.0 terminated with 24 4-(trimethylammoniummethyl)benzamide groups (90mg).  $^1\text{H}$  nmr ( $\text{D}_2\text{O}$ ) :  $\delta$  1.88; 2.65-2.80; 2.98; 3.10-3.60; 7.52 (br d,  $J=9\text{Hz}$ ); 7.72 (br d,  $J=9\text{Hz}$ ).  $^{13}\text{C}$  nmr ( $\text{D}_2\text{O}$ ) :  $\delta$  26.6; 33.4; 38.8; 43.2; 43.5; 53.6; 53.6; 54.1; 56.8; 62.8; 73.0; 132.1; 135.3; 137.5; 140.0; 176.4; 176.9; 183.6.

5

---

### EXAMPLE 17

#### Preparation of N-(2-Acetoxyethyl)-N,N-(dimethylammonium)methyl-carboxamide terminated dendrimers

10

PAMAM 4.0

Solid 1,1'-carbonyldiimidazole (85mg; 0.52mmol) was added to a solution of N-(2-acetoxyethyl)-N-(carboxymethyl)-N,N-dimethylammonium bromide (135mg; 0.5mmol) in dry DMF (3ml) and the resulting solution stirred under nitrogen for two hours. A solution of PAMAM 4.0 (60mg; 0.012mmol) in DMF (2ml) was then added, which caused the immediate formation of a flocculant precipitate which slowly redissolved. The mixture was stirred for two days and then concentrated ( $10^{-4}$  mmHg;  $40^\circ$ ) to give a viscous oil. The crude product was purified by gel filtration (Sephadex G10; 10% AcOH) to give PAMAM 4.0 terminated with 24 N-(2-Acetoxyethyl)-N,N-(dimethylammonium)methylcarboxamide groups (64mg).  $^1\text{H}$  nmr ( $\text{D}_2\text{O}$ ) :  $\delta$  1.93; 2.05; 2.70; 3.10-3.60; 3.28; 3.93 (m); 4.14; 4.48 (m).  $^{13}\text{C}$  nmr ( $\text{D}_2\text{O}$ ) :  $\delta$  24.6; 26.2; 33.2; 38.7; 42.8; 42.9; 53.9; 57.4; 62.6; 67.3; 67.5; 168.9; 176.4; 176.8; 177.3; 183.2.

25

**EXAMPLE 18****Preparation of Guanidino terminated dendrimers****5 PAMAM 4.0 BRI6042**

- A solution of PAMAM 4.0 (63mg; 0.012mmol) and methylthiopseudourea sulfate (170mg; 0.61mmol) in water (5ml) (pH 10.5) was heated under nitrogen at 80<sup>o</sup> for two hours. The solution was then concentrated and the residue purified by gel
- 10 filtration (Sephadex G10; 10% AcOH) to give PAMAM 4.0 terminated with 24 guanidino groups as the acetate salt (107mg). <sup>1</sup>H nmr (D<sub>2</sub>O) : δ 2.00; 2.80 (br t); 3.09 (br t); 3.32; 3.45 (br t); 3.60 (br t). <sup>13</sup>C nmr (D<sub>2</sub>O) : δ 25.2; 33.2; 33.4; 38.7; 41.2; 42.6; 43.4; 44.7; 53.5; 54.0; 56.3; 176.5; 176.7; 176.9; 181.6.
- 15 The corresponding PAMAM 2.0 dendrimer terminated with 6 guanidino groups was similarly prepared.

**EXAMPLE 19****20 Preparation of 4-([1,4,8,11-tetraazacyclotetradecane]methyl)benzamide terminated dendrimers**

---

**PAMAM 4.0 BRI6041**

- 25 A solution of 1-(4-carboxyphenyl)methyl-1,4,8,11-tetraazacyclotetradecane tetra hydrochloride (120mg; 0.25mmol), N-hydroxysuccinimide (60mg; 0.52mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (250mg; 1.3mmol) in pH 7 phosphate buffer (10ml) was allowed to stand a room temperature for one hour and then a solution of PAMAM 4.0 (32mg; 0.006mmol) in pH 7 phosphate buffer
- 30 (10ml) added. The mixture was allowed to stand for two days and then concentrated.

- 35 -

The residue was purified by gel filtration (Sephadex LH20; 10% AcOH) to give PAMAM 4.0 terminated with ca. 12 4-([1,4,8,11-tetraazacyclotetradecane]methyl)-benzamide groups as determined by  $^1\text{H}$  and  $^{13}\text{C}$  nmr (80mg). The product was then dissolved in water and passed through a column of Amberlite IRA-401 (Cl) resin and  
 5 then concentrated. The residue was dissolved in water (1ml), concentrated HCl (1ml) added, and the solution diluted with ethanol (30ml) to precipitate a white solid. The solid was collected by filtration (68mg). Once again  $^1\text{H}$  and  $^{13}\text{C}$  nmr showed ca. 50% functionalisation of the terminal amino groups.  $^1\text{H}$  nmr ( $\text{D}_2\text{O}$ ) :  $\delta$  2.17; 2.36; 2.50; 2.78; 2.85; 3.25; 3.40; 3.50; 3.60; 3.62; 4.49; 7.63 (br d); 7.78 (br d).  $^{13}\text{C}$  nmr  
 10 ( $\text{D}_2\text{O}$ ) :  $\delta$  22.7; 23.1; 33.2; 38.8; 39.9; 40.2; 40.3; 41.0; 41.2; 42.0; 42.9; 43.2; 43.6; 45.5; 46.1; 49.1; 52.2; 53.9; 54.3; 56.6; 62.7; 132.5; 135.7; 137.1; 139.7; 174.3; 176.2; 176.3; 176.7; 177.0; 178.2; 178.5.

## EXAMPLE 20

15

### Preparation of 4-Carboxy-3-hydroxybenzylamine terminated dendrimers

PAMAM 4.0 (EDA) **BRI6119**

20 Sodium cyanoborohydride (32mg; 0.5mmol) was added to a mixture of PAMAM 4.0 (EDA) (69mg; 0.01mmol), 4-formyl-2-hydroxybenzoic acid (83mg; 0.5mmol), and sodium hydrogen carbonate (42mg; 0.5mmol) in water (4ml). The inhomogeneous orange mixture was stirred for four hours at room temperature, during which time it became homogeneous. The orange solution was then concentrated and the residue  
 25 purified by gel filtration (Sephadex LH20; water) to give PAMAM 4.0 (EDA) terminated with ca. 32 4-carboxy-3-hydroxybenzylamine groups (91mg).  $^1\text{H}$  and  $^{13}\text{C}$  nmr ( $\text{D}_2\text{O}$ ) shows mostly mono alkylation but with some signs of dialkylation of the terminal amino groups, both spectra show broad peaks.  $^{13}\text{C}$  nmr ( $\text{D}_2\text{O}$ ) :  $\delta$  37.0;

- 36 -

41.1; 50.9; 53.4; 55.5; 55.8; 61.5; 120.9; 122.2; 122.4; 132.3; 132.7; 135.0; 135.8;  
163.5; 163.7; 169.0; 178.6; 179.3.  $^1\text{H}$  nmr ( $\text{D}_2\text{O}$ ) :  $\delta$  2.20; 2.35; 2.60; 3.15; 3.30;  
3.55; 4.25; 6.68; 7.12; 7.55.

5

**EXAMPLE 21**

---

**Preparation of 4-Carboxyphenylamide terminated dendrimers**

PAMAM 4.0 (EDA)

10

Solid 4-carboxyphenylisothiocyanate (86mg; 0.48mmol) was added to a solution of PAMAM 4.0 (EDA) (69mg; 0.01mmol) in water (20ml). The pH of the resulting cloudy solution was adjusted to 9 with saturated  $\text{NaHCO}_3$  solution and left to stir at room temperature for 24 hours. The reaction mixture was then filtered and the filtrate concentrated to give a white solid residue, which was purified by gel filtration (Sephadex LH20; water) and then freeze dried to give the product as a white fluffy solid (68mg).

15

**EXAMPLE 22**

20

**Preparation of 3,5-Dicarboxyphenylamide terminated dendrimers**

---

PAMAM 4.0 (EDA)

25 Solid 3,5-dicarboxyphenylisothiocyanate (112mg; 0.5mmol) was added to a solution of PAMAM 4.0 (EDA) (70mg; 0.01mmol) in water (5ml). The pH of the resulting cloudy solution was adjusted to 10 with 1M  $\text{Na}_2\text{CO}_3$  solution and heated under nitrogen at  $53^\circ$  for 2 hours. The reaction mixture was then filtered and the filtrate

- 37 -

concentrated to give a brownish solid residue, which was purified by gel filtration (Sephadex LH20; water) and then freeze dried to give the product as a pale brown solid (112mg).

5

**EXAMPLE 23****Preparation of Sodium 4-Phosphonooxyphenylthiourea terminated dendrimers**

PAMAM 4.0 (EDA)

10

Solid sodium 4-phosphonooxyphenylisothiocyanate (251mg) was added to a solution of PAMAM 4.0 (EDA) (69mg; 0.01mmol) in water (20ml). The resulting solution (pH 9) was stirred for 24 hours at room temperature under nitrogen. The reaction mixture was then concentrated to give a white solid residue, which was purified by gel filtration (Sephadex LH20; water) and then freeze dried to give the product as a fluffy white solid (86mg).

**EXAMPLE 24**

20

**Preparation of Sodium 4-(Phosphonomethyl)phenylthiourea terminated dendrimers**

PAMAM 4.0 (EDA)

25

Solid sodium 4-(phosphonomethyl)phenylisothiocyanate (97mg) was added to a solution of PAMAM 4.0 (EDA) (69mg; 0.01mmol) in water (30ml). The resulting solution was stirred for 3 days at room temperature under nitrogen, maintaining the pH at 8 with periodic addition of saturated  $\text{NaHCO}_3$  solution. The reaction mixture was then concentrated to give a white solid residue, which was purified by gel

- 38 -

filtration (Sephadex LH20; water) and then freeze dried to give the product as a fluffy white solid (102mg).

### EXAMPLE 25

5

#### **Preparation of Sodium Ethyl 4-(Phosphonomethyl)phenylthiourea terminated dendrimers**

---

PAMAM 4.0 (EDA)

10

Solid sodium ethyl 4-(phosphonomethyl)phenylisothiocyanate (109mg) was added to a solution of PAMAM 4.0 (EDA) (69mg; 0.01mmol) in DMF (30ml). The resulting solution was stirred for 17 hours at room temperature under nitrogen, maintaining the pH at 8 with periodic addition of saturated NaHCO<sub>3</sub> solution. The reaction mixture was then concentrated to give a white solid residue, which was purified by gel filtration (Sephadex LH20; water) and then freeze dried to give the product as a fluffy white solid (30mg).

### EXAMPLE 26

20

#### **Preparation of C<sub>n</sub>-alkyl linked 2-thiosialoside terminated dendrimers**

---

Methyl [(8-octanoic acid N-hydroxysuccinimide ester) 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-2-thio-D-glycero- $\alpha$ -D-galacto-2-nonulopyranosid]onate was prepared by the following procedure.

To a solution of methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-2-S-acetyl-3,5-dideoxy-2-thio-D-glycero- $\alpha$ -D-galacto-2-nonulopyranosonate (Hasegawa *et al*, 1986) (100mg.) in dry dimethylformamide (1ml) was added 8-bromooctanoic acid



- 39 -

(41mg.) and diethylamine (280mg.) and the solution stirred at 20° C for 17 hours. Solvent was removed under vacuum and the residue partitioned between ethyl acetate and ice cold 5% hydrochloric acid. The organic layer was washed with water, dried over sodium sulphate, and evaporated to give a residue (130mg.). This  
 5 was dissolved in ethyl acetate (5ml.) and N-hydroxysuccinimide (26mg.) and dicyclohexylcarbodiimide (46mg.) were added. The mixture was stirred at 20°C for 17 hours then the white precipitate was filtered off. The filtrate was concentrated and purified by flash chromatography on silica gel eluting with ethyl acetate. Fractions containing product were combined and evaporated to give a white foam  
 10 97mg. 71%.

Similarly were prepared:

Methyl [(11-undecanoic acid N-hydroxysuccinimide ester) 5-acetamido-4,7,8,9-  
 15 tetra-O-acetyl-3,5-dideoxy-2-thio-D-glycero- $\alpha$ -D-galacto-2-nonulopyranosid]onate.

Methyl [(acetic acid N-hydroxysuccinimide ester) 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-2-thio-D-glycero- $\alpha$ -D-galacto-2-nonulopyranosid]onate.

20 Methyl [(4-butanoic acid N-hydroxysuccinimide ester) 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-2-thio-D-glycero- $\alpha$ -D-galacto-2-nonulopyranosid]onate.

Methyl [(4-methylbenzoic acid N-hydroxysuccinimide ester) 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-2-thio-D-glycero- $\alpha$ -D-galacto-2-nonulopyranosid]onate.

25

A PAMAM [EDA] 4.0 [(8-octanamido)- 5-acetamido-3,5-dideoxy-2-thio-D-glycero- $\alpha$ -D-galacto-2-nonulopyranosidoic acid]<sub>32</sub> **BRI 6112**

To a solution of the PAMAM [EDA] 4.0 (50mg.) in dry dimethyl  
 30 sulphoxide(4ml.) under an inert atmosphere was added methyl [(8-octanoic

- 40 -

acid N-hydroxysuccinimide ester) 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-2-thio-D-glycero- $\alpha$ -D-galacto-2-nonulopyranosid]onate(300mg.) and the solution stirred for 60 hours at 20°C. The solvent was removed under vacuum and the residue was dissolved in methanol (2ml.). This solution was subjected to size exclusion chromatography on Sephadex LH20 eluting with methanol. On evaporation of solvent, the product, PAMAM [EDA] 4.0 [methyl [(8-octanamido) 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-2-thio-D-glycero- $\alpha$ -D-galacto-2-nonulopyranosid]onate]<sub>32</sub> was obtained as a white powder. 182mg. 93 %

This was converted to the free sialoside by the following method:

To a solution of PAMAM [ EDA ] 4.0 [methyl [(8-octanamido) 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-2-thio-D-glycero- $\alpha$ -D-galacto-2-nonulopyranosid]onate]<sub>32</sub> (182mg.) in dry methanol (3ml.) under argon at 20°C was added a freshly prepared 0.19M solution of sodium methoxide in methanol (7ml.) and the mixture stirred for 2.5 hours. The solvent was evaporated and the residue dissolved in water (10ml.) and stirred for 3 hours. This solution was subjected to size exclusion chromatography on Sephadex LH20 eluting with water. On lyophilisation, the product, PAMAM [EDA] 4.0 [(8-octanamido)- 5-acetamido-3,5-dideoxy-2-thio-D-glycero- $\alpha$ -D-galacto-2-nonulopyranosidoic acid]<sub>32</sub> was obtained as a pale lemon powder 110mg. 77%

By a similar procedure were prepared:

PAMAM [EDA] 4.0 [(11-undecanamido)-5-acetamido-3,5-dideoxy-2-thio-D-glycero- $\alpha$ -D-galacto-2-nonulopyranosidoic acid]<sub>32</sub> **BRI 6147**

PAMAM [EDA] 4.0 [ (acetamido)- 5-acetamido-3,5-dideoxy-2-thio-D-

- 41 -

glycero- $\alpha$ -D-galacto-2-nonulopyranosidoic acid]<sub>32</sub> **BRI 6121**

PAMAM [EDA] 4.0 [(4-methylbenzamido)- 5-acetamido-3,5-dideoxy-2-thio-D-glycero- $\alpha$ -D-galacto-2-nonulopyranosidoic acid]<sub>32</sub> **BRI 6120**

5

**B** BHA lyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub> [(8-octanamido)- 5-acetamido-3,5-dideoxy-2-thio-D-glycero- $\alpha$ -D-galacto-2-nonulopyranosidoic acid]<sub>32</sub> **BRI 6169**

10 A solution of BHA lyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub> (t-Boc)<sub>32</sub> (20.3mg.) in a mixture of trifluoroacetic acid (2ml.) and dichloromethane (2ml.) was stirred at 20°C for 2 hours then solvent was removed under vacuum. The residue was dissolved in dry dimethyl sulfoxide (1ml.) and di-isopropylethylamine (25mg.) and methyl [(8-octanoic acid N-hydroxysuccinimide ester) 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-2-thio-D-glycero- $\alpha$ -D-galacto-

15 2-nonulopyranosid]onate (78mg.) were added. The mixture was stirred under argon at 20°C for 60 hours then solvent was removed under vacuum. The residue was dissolved in a freshly prepared 0.1M solution of sodium methoxide in methanol (2.5ml.) and the mixture stirred for 3 hours under argon at 20°C. The solvent was evaporated and the residue dissolved in

20 water (1ml.) and stirred for 17 hours . This solution was subjected to size exclusion chromatography on Sephadex LH20 eluting with water. After lyophilisation ,the product, BHA lyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub> [(8-octanamido)- 5-acetamido-3,5-dideoxy-2-thio-D-glycero- $\alpha$ -D-galacto-2-nonulopyranosidoic acid]<sub>32</sub> was obtained as a white powder 44mg. 86%.

25

**EXAMPLE 27****Preparation of dendritic sialosides modified in the 4-position of sialic acid**

5 Methyl 4-azido-5-acetamido-7,8,9-tri-O-acetyl-2-S-acetyl-3,4,5-trideoxy-2-thio-D-glycero- $\alpha$ -D-galacto-2-nonulopyranosonate was prepared by the following procedure. To a solution of methyl 4-azido-5-acetamido-7,8,9-tri-O-acetyl-2-chloro-3,4,5-trideoxy-D-glycero- $\beta$ -D-galacto-2-nonulopyranosonate (Sabesan, 1994) (5g.) in dry dichloromethane (150ml.) was added finely powdered potassium  
10 thiolacetate (5.8g.) and the suspension stirred vigorously at 20°C for 48 hours. The mixture was filtered and evaporated to give a light brown foam (5.2g.). The required product was isolated by preparative reversed phase HPLC [ $C_{18}$ , 30% acetonitrile/water] as a white foam 3.9g. 72%.

15 Methyl [(8-octanoic acid N-hydroxysuccinimide ester) 4-azido-5-acetamido-7,8,9-tri-O-acetyl-3,4,5-trideoxy-2-thio-D-glycero- $\alpha$ -D-galacto-2-nonulopyranosid]onate was prepared by the following procedure.

To a solution of methyl 4-azido-5-acetamido-7,8,9-tri-O-acetyl-2-S-acetyl-3,4,5-  
20 trideoxy-2-thio-D-glycero- $\alpha$ -D-galacto-2-nonulopyranosonate (300mg.) in dry dimethylformamide (3.5ml.) was added 8-bromooctanoic acid (155mg.) and diethylamine (1.26ml.) and the solution stirred at 20° C for 17 hours. Solvent was removed under vacuum and the residue partitioned between ethyl acetate and ice  
cold 10% hydrochloric acid. The organic layer was washed with water, dried over  
25 sodium sulphate, and evaporated to give a yellow foam (385mg.). This was dissolved in ethyl acetate (20ml.) and N-hydroxysuccinimide (95mg.) and dicyclohexylcarbodiimide (175mg.) were added. The mixture was stirred at 20°C for 17 hours then the white precipitate was filtered off. The filtrate was concentrated and purified by preparative reversed phase HPLC [ $C_{18}$ , 30%  
30 acetonitrile/water] to give a white foam 340mg. 83%.

- 43 -

A PAMAM [EDA] 4.0 [ (8-octanamido)- 4-azido-5-acetamido-3,4,5-trideoxy-2-thio-D-glycero- $\alpha$ -D-galacto-2-nonulopyranosidoic acid]<sub>32</sub> **BRI 6146**

To a solution of the PAMAM [EDA] 4.0 (72mg.) in dry dimethyl sulphoxide (5ml.) under an inert atmosphere was added methyl [(8-octanoic acid N-hydroxysuccinimide ester) 4-azido-5-acetamido-7,8,9-tri-O-acetyl-3,4,5-trideoxy-2-thio-D-glycero- $\alpha$ -D-galacto-2-nonulopyranosid]onate (318 mg ) and the solution stirred for 60 hours at 20°C. The solvent was removed under vacuum and the residue was dissolved in methanol (2ml.). This solution was subjected to size exclusion chromatography on Sephadex LH20 eluting with methanol. On evaporation of solvent, the product, PAMAM [EDA] 4.0 [methyl [(8-octanamido) 4-azido-5-acetamido-7,8,9-tri-O-acetyl-3,4,5-trideoxy-2-thio-D-glycero- $\alpha$ -D-galacto-2-nonulopyranosid]onate]<sub>32</sub> was obtained as a white foam. 225mg. 81 %

The free sialoside was obtained by the following method:

To a solution of PAMAM [EDA] 4.0 [methyl [(8-octanamido) 4-azido-5-acetamido-7,8,9-tri-O-acetyl-3,4,5-trideoxy-2-thio-D-glycero- $\alpha$ -D-galacto-2-nonulopyranosid]onate]<sub>32</sub> (215mg.) in dry methanol (1ml.) under argon at 20°C was added a freshly prepared 1M solution of sodium methoxide in methanol (1ml.) and the mixture stirred for 3 hours. The solvent was evaporated and the residue dissolved in water (2ml.) and stirred for 17 hours. This solution was subjected to size exclusion chromatography on Sephadex LH20 eluting with water. On lyophilisation, the product, PAMAM [EDA] 4.0 [(8-octanamido)- 4-azido-5-acetamido-3,4,5-trideoxy-2-thio-D-glycero- $\alpha$ -D-galacto-2-nonulopyranosidoic acid]<sub>32</sub> was obtained as a fluffy white powder 160mg. 90%

30 B PAMAM [EDA] 4.0 [(8-octanamido)- 4-amino-5-acetamido-3,4,5-trideoxy-

- 44 -

2-thio-D-glycero- $\alpha$ -D-galacto-2-nonulopyranosidoic acid]<sub>32</sub> **BRI 6149**

A slow stream of hydrogen sulphide gas was passed into a solution of  
 PAMAM [EDA] 4.0 [(8-octanamido)- 4-azido-5-acetamido-3,4,5-trideoxy-  
 5 2-thio-D-glycero- $\alpha$ -D-galacto-2-nonulopyranosidoic acid]<sub>32</sub> (25mg.) in a  
 mixture of pyridine (40ml.) and water (20ml.) at 20°C for 5 days. The  
 solution was then bubbled with nitrogen for 2 hours to remove excess  
 hydrogen sulphide. The solution was evaporated to dryness and the residue  
 taken up in water (5 ml) and filtered through a 0.45 $\mu$ m. membrane filter to  
 10 remove sulphur. On lyophilisation, the product, PAMAM [EDA] 4.0 [(8-  
 octanamido)- 4-amino-5-acetamido-3,4,5-trideoxy-2-thio-D-glycero- $\alpha$ -D-  
 galacto-2-nonulopyranosidoic acid]<sub>32</sub> was obtained as a fluffy white powder  
 23mg. 96%

**EXAMPLE 28**

**Preparation of boronic acid terminated dendrimers.**

4-Carboxyphenylboronic acid N-hydroxysuccinimide ester

20 To a solution of 4-carboxyphenylboronic acid (500mg.) in dry dimethyl formamide  
 (5ml) were added N-hydroxysuccinimide (380mg.) and dicyclohexylcarbodiimide  
 (680mg) The mixture was stirred at 20° C for 64 hours then the white precipitate  
 was filtered off. The solvent was removed under vacuum and the residue dissolve  
 25 in ethyl acetate (100ml.). This solution was washed with water, dried over sodium  
 sulphate and evaporated to give a white solid which was crystallised from  
 acetonitrile/water as fine needles 730mg. 92%

- 45 -

PAMAM [EDA] 4.0 [4-benzamidoboronic acid]<sub>32</sub> **BRI 6160**

To a solution of the PAMAM [EDA] 4.0 (69mg.) in dry dimethyl sulphoxide (5ml) under an inert atmosphere was added 4-carboxyphenylboronic acid N-

5 hydroxysuccinimide ester (130mg.) and the solution stirred for 65 hours at 20°C.

To the thick slurry was added 1M sodium carbonate solution (1ml.) and the clear solution stirred an additional 24 hours. The solvent was removed under vacuum and the residue was dissolved in 10% ammonia solution (5ml.). This solution was subjected to size exclusion chromatography on Sephadex LH20 eluting with 10%  
10 ammonia solution . On evaporation of solvent, the product, PAMAM [EDA] 4.0 [4-benzamidoboronic acid]<sub>32</sub> was obtained as a white fluffy solid. 110mg. 94%.

### EXAMPLE 29

15 **Preparation of Sodium 3,6-disulfonaphthylthiourea terminated dendrimers.**

BHAlyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub>lys<sub>32</sub>

Trifluoroacetic acid (2ml) was added to a stirred suspension of

20 BHAlyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub>DBL<sub>32</sub> (147mg) in dry dichloromethane (2ml) and the resulting solution stirred at room temperature under nitrogen for two hours and

then concentrated. The residue was dissolved in N,N-dimethyl-N-allylamine buffer (pH 9.5; 5ml) and then solid 3,6-disulfonaphthyl isothiocyanate (400mg) added.

The pH of the mixture was then adjusted to 9.5 by the addition of 1M sodium

25 carbonate and the solution heated at 53°C for three hours under nitrogen. The

reaction mixture was concentrated and the residue redissolved in water and the solution passed through a column of Amberlite IR 120 (Na). The filtrate was

concentrate was concentrated to give the crude product, which was purified by gel filtration (Sephadex LH20; water) to give BHAlyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub>lys<sub>32</sub> with 64

30 sodium 3,6-disulfonaphthylurea groups as a white fluffy solid (175mg).

**EXAMPLE 30****Preparation of Sodium 3,5-Disulfophenylthiourea terminated dendrimers.**

5

BHAlyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub>lys<sub>32</sub>

Trifluoroacetic acid (3ml) was added to a stirred suspension of BHAlyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub>DBL<sub>32</sub> (300mg; 0.02mmol) in dry dichloromethane (3ml) and the resulting solution stirred at room temperature under nitrogen for two hours and then concentrated. The residue was dissolved in water and the solution passed through a column of Amberlite IRA 401 (OH) and the filtrate concentrated to give a viscous oil (187mg). The oil was dissolved in a 1:1 mixture of pyridine/water (8ml) and solid sodium 3,5-disulfophenyl isothiocyanate (680mg; 2mmol) added. The resulting solution was heated at 53°C for three hours under nitrogen. The solution was then concentrated to give a white solid residue. The crude product was purified by gel filtration (Sephadex LH20; water) to give BHAlyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub>lys<sub>32</sub> with 64 sodium 3,6-disulfophenylurea groups as a white fluffy solid.

20

**EXAMPLE 31****Preparation of Sodium 3,5-Dicarboxyphenylthiourea terminated dendrimers.**25 BHAlyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub>lys<sub>32</sub>

Trifluoroacetic acid (3ml) was added to a stirred suspension of BHAlyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub>DBL<sub>32</sub> (300mg; 0.02mmol) in dry dichloromethane (3ml) and the resulting solution stirred at room temperature under nitrogen for two hours and then concentrated. The residue was dissolved in water and the solution passed

30



- 47 -

through a column of Amberlite IRA 401 (OH) and the filtrate concentrated to give a viscous oil (186mg). The oil was dissolved in a 1:1 mixture of pyridine/water (8ml) and sodium 3,5-dicarboxyphenyl isothiocyanate (450mg; 2mmol) added. The resulting solution was heated at 53°C for 13 hours under nitrogen. The solution  
 5 was then concentrated to give a white solid residue. The crude product was purified by gel filtration (Sephadex LH20; water) to give BHAlyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub>lys<sub>32</sub> with 64 sodium 3,6-dicarboxyphenylurea groups as a white fluffy solid.

10

**EXAMPLE 32****Preparation of Sodium 4-phosphonooxyphenylthiourea terminated dendrimers.**BHAlyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub>lys<sub>32</sub>

15

Trifluoroacetic acid (2ml) was added to a stirred suspension of BHAlyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub>DBL<sub>32</sub> (147mg; 0.01 mmol) in dry dichloromethane (2ml) and the resulting solution stirred at room temperature under nitrogen for two hours and then concentrated to give a viscous oil. The oil was dissolved in N,N-  
 20 dimethyl-N-allylamine buffer (pH 9.5; 5ml) and solid 4-phosphonooxyphenyl isothiocyanate (250mg) added. The pH of the resulting solution was adjusted to 10 with 1M sodium carbonate and the mixture heated at 53°C for three hours under nitrogen. The solution was then concentrated to give a white solid residue. The residue was redissolved in water and the solution passed through a column of  
 25 Amberlite IR 120 (Na) and the filtrate concentrated. The residue was then purified by gel filtration (Sephadex LH20; water) to give BHAlyslys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub>lys<sub>32</sub> with 64 sodium 4-phosphonooxyphenylurea groups as a white fluffy solid (150mg).

**EXAMPLE 33****Preparation of Sodium 4-phosphonophenylthiourea terminated dendrimers.**

5 BHAl<sub>2</sub>lys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub>lys<sub>32</sub>

Trifluoroacetic acid (2ml) was added to a stirred suspension of  
 BHAl<sub>2</sub>lys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub>DBL<sub>32</sub> (147mg; 0.01 mmol) in dry dichloromethane (2ml)  
 and the resulting solution stirred at room temperature under nitrogen for two hours  
 10 and then concentrated to give a viscous oil. The oil was dissolved in N,N-  
 dimethyl-N-allylamine buffer (pH 9.5; 5ml) and solid 4-phosphonophenyl  
 isothiocyanate (250mg) added. The pH of the resulting solution was adjusted to 9  
 with saturated sodium bicarbonate solution and the mixture heated at 53°C for  
 three hours under nitrogen. The solution was then concentrated to give a white  
 15 solid residue. The residue was redissolved in water and the solution passed  
 through a column of Amberlite IR 120 (Na) and the filtrate concentrated. The  
 residue was then purified by gel filtration (Sephadex LH20; water) to give  
 BHAl<sub>2</sub>lys<sub>2</sub>lys<sub>4</sub>lys<sub>8</sub>lys<sub>16</sub>lys<sub>32</sub> with 64 sodium 4-phosphonophenylurea groups  
**BRI 6196** as a white fluffy solid (152mg) after freeze drying.

20

**EXAMPLE 34****Preparation of Sodium 4,6-diphosphononaphthylthiourea terminated dendrimers.**

25

PAMAM 4.0

A solution of sodium 4,6-diphosphononaphthyl isothiocyanate (165mg) in water  
 (2ml) was added to a solution of PAMAM 4.0 (51mg; 0.01mmol) in water (2ml).  
 30 The pH of the mixture was adjusted to 9.5 with saturated sodium bicarbonate

- 49 -

solution and the mixture vigorously stirred for one hour at room temperature and then heated at 53°C for three hours under nitrogen. The mixture was then filtered and the filtrate concentrated to give a brown solid residue. The crude product was purified by gel filtration (Sephadex G25; water) to give PAMAM 4.0 terminated  
5 with 24 sodium 4,6-diphosphononaphthylthiourea groups as a brown solid (81mg) after freeze drying.

---

### EXAMPLE 35

#### 10 Preparation of Fluoresceinthiourea terminated dendrimers.

##### PAMAM 4.0 (EDA)

Solid fluorescein isothiocyanate (188mg) was added to a solution of PAMAM 4.0  
15 (EDA) (74mg; 0.01mmol) in water (3ml). Saturated sodium bicarbonate solution was added to adjust the pH to 9 and the resulting homogenous solution stirred overnight at room temperature and then concentrated. The orange residue was purified by gel filtration (Sephadex LH20; water) to give PAMAM 4.0 (EDA) terminated with 21 fluoresceinthiourea groups as a fluffy orange solid (193mg)  
20 after freeze drying.

### EXAMPLE 36

---

#### Preparation of Sodium (phenyl-3-boronic acid)-thiourea terminated dendrimers.

25

##### PAMAM 4.0 (EDA)

Solid (phenyl-3-boronic acid) isothiocyanate (100mg; 0.5mmol) was added to a solution of PAMAM 4.0 (EDA) (69mg; 0.01mmol) in water (5ml). 1M sodium  
30 carbonate was added to the isothiocyanate dissolved (pH ca.10). The mixture was

- 50 -

then heated at 53°C for two hours under nitrogen, and then filtered and the filtrate concentrated to give a brownish solid residue. The crude product was purified by gel filtration (Sephadex LH20; water) to give PAMAM 4.0 (EDA) terminated with 32 (phenyl-3-boronic acid)thiourea groups as a white fluffy solid (87mg) after freeze drying.

---

### EXAMPLE 37

#### A Materials and Methods

10

##### *Cytosensor Microphysiometer Protocol*

The Cytosensor Microphysiometer (Molecular Devices Inc., CA) is a light addressable potentiometric sensor-based device that can be used to indirectly measure the metabolic rate of cells *in vitro* (Parce *et al.*, 1989; McConnell *et al.*, 1992). Metabolism is determined by measuring the rate of acid metabolite production from cells immobilised inside a microvolume flow chamber.

Human CEM cells were centrifuged and resuspended in low-buffered serum-free/bicarbonate-free RPMI 1640 medium (Molecular Devices; hereafter referred to as "modified medium"). The cells were seeded at a density of 60,000-75,000 cells/capsule onto the polycarbonate membrane (3 µm porosity) of cell capsule cups (Molecular Devices). Cells were immobilised using an agarose entrapment medium (Molecular Devices). The seeded capsule cups were transferred to sensor chambers containing the silicon sensor which detects changes in pH (and thus cellular metabolism). The Cytosensor system used for this set of experiments contained eight separate chambers for the measurement of acidification rates. Modified media was pumped across the cells at a rate of 100-120 µl/min. Each cell chamber was served by fluid from either of two reservoirs, which could be alternated using a software command.

- 51 -

To measure the acidification rate, flow of the modified media was periodically interrupted, allowing the accumulation of excreted acid metabolites (lactic acid and CO<sub>2</sub>). In this set of experiments, flow was stopped for 30s, during which time, a least squares fit slope to the change in voltage signal over time, the acidification rate (measured as  $\mu$  V/s), was calculated. This rate data was normalised (using the 4-5 rate points prior to addition of compound) to allow direct comparison of the signals from the four chambers. Measurements of the acidification rate were made every 2 min. The chamber was held at 37°C.

10 Basal acidification rates were monitored (in the absence of any treatment) for at least 30 min. After this time, the venoms/peptides were exposed to the cells at a range of concentrations for periods of up to 4hrs. A concentration of toxin showing a pronounced effect on the cells, but less than maximal, was selected for testing of inhibition of this toxicity by a range of concentrations of BRI2923. In all 15 experiments, at least one chamber was not exposed to any of the compounds, providing a negative control.

BRI2923 was dissolved in water and the solution was pH adjusted to 7.2.

Concentrations ranging from 100  $\mu$ M to 1 nM were added to the venom/media 20 solutions and incubated for periods ranging from 6 min (the minimum incubation period possible using this equipment) to 1 hr and then introduced to the cells. All experiments were repeated to a minimum of n=3.

## B Results

25

### *BRI2923 Inhibition of Cobra Venom.*

Crude venom from the forest cobra (*Naja malenoleuca*) was tested. Cobra venom added to CEM cells caused an initial increase in cellular metabolism followed by 30 cell death (presumed cell lysis). Cobra venom was particularly damaging to the

- 52 -

cells causing an initial increase in metabolism of approx. 80% followed rapidly by 100% cell death within the first 10 minutes. The venoms were initially tested at 10, 50 and 100  $\mu\text{g/ml}$  were the submaximal response from 50  $\mu\text{g/ml}$  was used as the test dose in each experiment. Two concentrations of BRI2923 ( $10^{-4}\text{M}$  and  
 5  $10^{-5}\text{M}$ ) were each incubated with 50  $\mu\text{g/ml}$  venom.

$10^{-5}\text{M}$  BRI2923, at all incubation periods: 6, 30 and 60 mins, reduced the initial increase in metabolic rate from approx. 80% to approx. 5% and delayed the onset of cell death by approx. 15-20 minutes.

10

$10^{-4}\text{M}$  BRI2923, incubated with 50  $\mu\text{g/ml}$  venom, blocked both the initial increase in metabolic rate as well as the subsequent rapid cell death seen at this concentration of venom alone. The cells were, however, less robust than those with  $10^{-4}\text{M}$  BRI2923 alone.  $10^{-4}\text{M}$  BRI2923, incubated with 100  $\mu\text{g/ml}$  venom also  
 15 abolished the initial increase in metabolic rate but the cells proceeded to cell death (albeit more slowly) after the venom/dendrimer solution was washed out.

However,  $10^{-4}\text{M}$  BRI2923 has a buffering effect which is seen in the cytosensor system as an initial decrease in metabolic rate which is reversed on washout. This result is particularly interesting as all snake venoms consist of many toxic

20 components which have different modes of toxicity. From the literature, all cobra venoms contain a cytotoxic peptide which causes cell lysis in a way similar to melittin. The amino acid sequence of the cytotoxin isolated from *Naja malenoleuca*  
 indicates that this toxin is highly basic (cationic) and would thus be readily  
 inactivated by polyanionic compounds such as BRI2923. This electrostatic  
 25 inactivation as a basis for reduced toxicity is supported by the experimental finding that an incubation period of 6 minutes gives the same result as the longer incubation periods of 30-60 mins used.

*BRI2923 Inhibition of Melittin (major toxin from bee venom).*

30

- 53 -

Initially, melittin was added to the CEM cells in half log doses ranging from  $10^{-5}\text{M}$  to  $10^{-7}\text{M}$ . The two highest doses ( $10^{-5}\text{M}$  and  $5 \times 10^{-6}\text{M}$ ) caused total cell death within 15 mins with no initial activation of the cells.  $10^{-6}\text{M}$  caused a transient increase in cell metabolic rate followed by cell lysis, complete after approx. 1 hr.

- 5  $5 \times 10^{-7}\text{M}$  caused the lysis curve to shift further to the right and  $10^{-7}\text{M}$  was without effect. This dose response determination was repeated and  $10^{-6}\text{M}$  melittin was selected as the submaximal concentration to be used with BRI2923. A range of concentrations of BRI2923 were incubated with  $10^{-6}\text{M}$  melittin for 20 mins.  $10^{-4}\text{M}$  and  $10^{-5}\text{M}$  BRI2923 completely inhibited the toxic effects of the melittin (note:
- 10 BRI2923 has an intrinsic buffering effect of its own at these concentrations which appears to lower the metabolic rate, this is not a real effect and reverses on washout)  $10^{-6}\text{M}$  BRI2923 completely blocks the melittin toxicity for approx. 30 mins and during the final 30 mins of exposure the metabolic rate only falls by approx. 10-15% more than the control cells.  $10^{-7}\text{M}$ ,  $5 \times 10^{-7}\text{M}$  and  $10^{-8}\text{M}$
- 15 BRI2923 shift the toxicity curve to the right in a dose-dependent fashion (i.e. reduces, but does not prevent, toxicity).  $10^{-9}\text{M}$  BRI2923 has no effect on  $10^{-6}\text{M}$  melittin.
-

## REFERENCES

McConnell, H.K., Owicki, J.C., Parce, J.W., Miller, D.L., Baxter, G.T., Wada, H.G. and Pitchford, S. (1992). The cytosensor microphysiometer: biological applications of silicon technology. *Science*, **257**, 1901-1912.

Parce, J.W., Owicki, J.C., Kercso, K.M., Sigal, G.B., Wada, H.G., Muir, V.C., Bousse, L.J., Ross, K.L., Sikic, B.I. and McConnell, H.M. (1989). Detection of cell-affecting agents with a silicon biosensor. *Science*, **246**, 243-247.

Dated this 14th day of September 1998

Starpharma <sup>Limited</sup> ~~Pty.Ltd.~~

By its Patent Attorneys  
*Davies Collison Cave*

